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(54) Title: PLANT TRANSCRIPTION REGULATORS FROM CIRCOVIRUS

(57) Abstract

The present invention is directed to transcription regulators and transcription regulator-like sequences of circovirus origin. As used in the specification, the circovirus group is considered to include subterranean clover stunt virus (SCSV) coconut foliar decay virus (CFDV), banana bunchy top virus (BBTV), milk-vetch dwarf virus (MDV) and faba bean necrotic yellow virus (FBNYV). The transcription regulators and transcription regulator-like sequences of the instant invention are useful in genetic engineering of plants and in particular leguminous plants such as to facilitate or control expression of foreign genes. The transcription regulators and transcription regulator-like sequences of the present invention are also useful in facilitating different levels of expression in different plant tissue types.

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PLANT TRANSCRIPTION REGULATORS FROM CIRCOVIRUS

The present invention relates generally to a novel range of transcription regulators and transcription regulator-like sequences operable in plants. More particularly, the present invention is directed to transcription regulators and transcription regulator-like sequences of viral origin and, even more particularly, of circovirus origin. The transcription regulators and transcription regulator-like sequences of the instant invention are useful in genetic engineering of plants and in particular leguminous plants such as to facilitate or control expression of foreign genes. The transcription regulators and transcription regulator-like sequences of the present invention are also useful in facilitating different levels of expression in different plant tissue types.

Bibliographic details of the publications referred by author in this specification are collected at the end of the description. Sequence Identity Numbers (SEQ ID Nos.) for the nucleotide sequences referred to in the specification are defined following the bibliography.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Transcription regulators are molecules, and generally nucleotide-based molecules, which facilitate and modulate expression of genetic sequences at the level of transcription. Transcription regulators include promoters and termination and polyadenylation sequences amongst other effectors and facilitators of transcription.

Promoters are specific nucleotide sequences to which RNA polymerase binds to initiate RNA synthesis in cells. They contain the start site for RNA synthesis and the genetic signals to initiate polymerase mediated RNA synthesis. In addition, sequence specific

DNA-binding proteins are presumed either to inhibit or to stimulate the initiation of RNA synthesis by binding next to the promoter and affecting the binding of the polymerase to the promoter. Terminator sequences refer to termination and polyadenylation sequences and are required for transcription of functional mRNAs. Termination sequences are located downstream (i.e. at the 3′ end) of a gene and are recognized by RNA polymerase as a signal to stop synthesizing mRNA. Polyadenylation sequences are signals required for polyadenylation of eukaryotic mRNA molecules following transcription.

A viral promoter widely used to facilitate foreign gene expression in plants is the cauliflower mosaic virus (CaMV) 35S promoter (Odell *et al.* 1985), which is derived from a double-stranded DNA plant virus. The use of this promoter in plants and plant cells is well documented (Benfey and Chua, 1990; Higgins and Spencer, 1991). However, despite the apparent usefulness of this promoter, it is not functional in all plants and is particularly poorly operable in leguminous plants. There is a need, therefore, to identify other promoters, such as of viral origin, which are operable in plants and particularly leguminous plants. There is also a need to modulate levels of expression of genes and other genetic sequences within plant cells.

Accordingly, one aspect of the present invention is directed to a genetic construct comprising a circovirus promoter or promoter-like sequence and which is operable in a plant cell.

In a related aspect of the present invention, there is provided a genetic construct comprising a circovirus promoter or promoter-like sequence and a termination and/or polyadenylation sequence, which sequences are operable in a plant cell.

A circovirus is a non-geminated single stranded (ss) DNA plant virus (Table 1), distinct from caulimoviruses which have a double stranded genome, and geminiviruses, the only other known ssDNA plant viruses, which have geminated particles (Chu *et al.*, 1994). As used herein, the circovirus group is considered to include subterranean clover stunt virus (SCSV) (Chu and Helms, 1988), coconut foliar decay virus (CFDV) (Rohde *et al.*

1990), banana bunchy top virus (BBTV) (Thomas and Dietzgen, 1991; Harding *et al.* 1991; 1993; Burns *et al.* 1993) and milk-vetch dwarf virus (MDV) (Isogai *et al.* 1992; Sano *et al.* 1993) and faba bean necrotic yellows virus (FBNYV) (Katul *et al.*, 1993).

In a particularly preferred embodiment, the circovirus contemplated for use in accordance with the present invention comprises more than two DNA components or segments.

The present invention is particularly directed and hereinafter described with reference to subterranean clover stunt virus (hereinafter abbreviated to "SCSV') as a representative of the circovirus group. This is done, however, with the understanding that reference to SCSV includes reference to all other suitable members of the circovirus group to which the instant invention extends. Preferred members of the circovirus group such as SCSV comprise more than two DNA components or segments. Reference hereinafter to "SCSV" also includes and extends to all naturally occurring or artificially induced mutants, derivatives, parts, fragments, homologues or analogues of the virus which still retain at least one suitable promoter and/or termination and/or polyadenylation sequences.

Accordingly, a particularly preferred embodiment of the present invention contemplates a genetic construct comprising an SCSV promoter or promoter-like sequence and which is operable in a plant cell.

A related aspect of the present invention is directed to a genetic construct comprising an SCSV promoter or promoter-like sequence and a termination and/or polyadenylation sequence, which sequences are operable in a plant cell.

The term "genetic construct" is used in its broadest sense to include any recombinant nucleic acid molecule such as an isolated nucleic acid molecule, vector, expression vector or binary vector. It may comprise solely the circovirus promoter or may contain one or more promoters in association with regulatory and/or reporter sequences.

The genetic construct may be double or single stranded DNA, in linear or covalently closed circular form. As stated above, it may comprise only the promoter or promoter-like sequence or may carry other heterologous or homologous transcription regulator sequences and/or heterologous structural gene sequences including promoters associated with SCSV. By "homologous" is meant a gene sequence naturally associated with the SCSV promoter. "Heterologous" means a "foreign" gene relative to the promoter or a gene not otherwise normally associated with the SCSV promoter. In a preferred embodiment, the foreign gene is also foreign to SCSV. Examples of foreign genes include genes which facilitate resistance to insects or other pest infestation, enhance resistance to insecticides or herbicides, promote frost resistance, alter flower or petal colour, decrease the rate of senescence, especially in cut flowers, increase or enhance levels of certain proteins and/or ribozymes. More particularly, the foreign genes include:

a) a resistance gene against plant viruses, bacteria, fungi, nematode and other pathogens;

b) a plant virus resistance gene including a synthetic gene from and against alfalfa mosaic virus, subterranean clover stunt virus, subterranean clover mottle virus, subterranean clover red leaf virus, potato leafroll virus, tomato spotted wilt virus, bean yellow mosaic virus, white clover mosaic virus, clover yellow vein virus, potato viruses x, y, s, m and a, cucumber mosaic virus, rice ragged stunt virus and barley yellow dwarf virus;

c) a gene to improve nutritional value of plants such as sunflower high sulphur gene SF8;

d) a bloat resistance gene;

e) an antibody gene;

f) a cereal thionin and ribosome inactivating protein gene;

g) an insect resistance gene including BT toxin gene and proteinase inhibitor gene from *Nicotiana alata*;

h) a selectable marker gene such as those conferring resistance to kanamycin, phosphinothricin, spectinomycin and hygromycin;

i) a reporter gene such as GUS, CAT and pigment genes;

j) a gene encoding a regulatory protein which modulates expression of a gene in plant cells.

The present invention further contemplates a genetic construct comprising two heterologous genes operably linked to the same or different circovirus promoters operable in a plant cell. Preferaby the promoter or different promoters are from a circovirus with a genome comprising more than two components or segments. Most preferably the promoter or different promoters are from SCSV and in particular are selected from segments 1 to 7 of SCSV as defined by SEQ ID NOs. 1 to 7. (See below). The genetic constructs may also comprise a termination or polyadenylation sequence operably linked to one or both of the heterologous genes. In one embodiment, the termination and/or polyadenylation sequence is the same for each gene. In an alternative embodiment the termination and/or polyadenylation sequence is different for each gene. Most preferably the termination and polyadenylation sequence is selected from segments 1 to 7 of SCSV as defined by SEQ ID NOs. 1 to 7 (See below). In yet another embodiment at least one termination and polyadenylation sequence is from the MeA 3 gene of *Flaveria bidentis* (see below).

The term "transcription regulator" is used in its broadest sense to include promoters, termination and polyadenylation sequences and other effectors and facilitators of transcription of genetic sequences. As with promoters contemplated by the present inveniton, the termination and polyadenylation sequences may be of SCSV origin and may be naturally associated with a corresponding promoter from SCSV or may be associated with another promoter of SCSV. Alternatively, the termination and/or polyadenylation sequences may be derived from non-SCSV sources. A particularly preferred terminator comprises the 3' nucleotide sequence of the MeA3 gene of *Flaveria bidentis* which codes for an NADP-malic enzyme of C4 photosynthesis (Hatchi, 1987). The nucleotide sequence of the terminator region of the MeA gene is shown in Figure 15. The combination of an SCSV promoter with, for example, the *F. bidentis* MeA gene terminator sequence results in a high level of expression especially in monocotyledonas plants relative to constructs without the terminator sequence.

The foreign gene may also be in the antisense orientation so as to facilitate reduced levels of endogenous plant gene products. In this regard, "gene" may be ten base pairs in length, tens of base pairs in length, hundreds of base pairs in length or a full length

or near full length gene but in a reverse orientation relative the promoter. The foreign gene may also be placed in the "sense" orientation for co-suppression of a target gene.

According to another aspect of the present invention, there is provided a genetic construct comprising an SCSV promoter or promoter-like sequence operable in a plant cell and at least one restriction endonuclease site downstream of said promoter to facilitate insertion of a heterologous gene such that said gene is operably linked to said promoter. In an alternative embodiment, the genetic construct comprises an SCSV promoter or promoter-like sequence operable in a plant cell and a heterologous gene operably linked to said promoter. In both embodiments, the term "gene" includes those directing the synthesis of oligonucleotides such as those useful in antisense techniques as well as ribozymes.

In still a further embodiment of the present invention, there is contemplated a genetic construct comprising an SCSV promoter or promoter-like sequence operable in a plant cell and at least one restriction endonuclease site downstream of said promoter to facilitate insertion of a heterologous gene such that said gene is operably linked to said promoter and a termination and/or polyadenylation sequence positioned such that same sequence is at the 3' end relative to said heterologous gene to facilitate expression of said heterologous gene. Preferably, the termination sequence is from SCSV. Alternatively, the terminator sequence is from the *F. bidentis* MeA3 gene.

Plants contemplated by the present invention include both monocotyledonous and dicotyledonous species. The present invention also extends to leguminous and non-leguminous plants although leguminous plants are preferred.

The SCSV genome comprises at least seven distinct circular ssDNA components described as segments 1-7. The size of these segments range from about 988 to about 1022 nucleotides. Each of the seven DNA components of SCSV contains one major open reading frame of the viral sense and a non-coding region of various lengths containing a conserved potential stem and loop structure (Figures 1 and 2; Table 2). Each transcription unit contains a typical TATA box and a polyadenylation signal for

start and end of transcription, respectively (Figure 2, Table 2).

Because the DNAs are circular, the sequences in the non-coding regions comprise the promoters and the terminator signals which vary with different DNA components (Table 2). The TATA boxes and the stem-loops of the two replicase-associated protein genes in segments 2 and 6 are quite different from those of the other genes. In contrast, the stem-loops and TATA boxes are the same in segments 1, 3, 4, 5 and 7. All the DNAs, except those of segments 2 and 6, also share a common sequence (known as the common region) in the non-coding region (Figures 1 and 2).

The present invention, therefore, extends to each of the seven promoters and to termination and polyadenylation sequences on segments 1-7 of SCSV. The nucleotide sequences of segments 1-7 are shown in Figure 6 and are defined in SEQ ID NOs. 1-7, respectively.

Segment 5 was identified as the coat protein gene based on N-terminal amino acid sequence and amino acid composition data (Chu *et al.*, 1993a). Segments 2 and 6 encode proteins containing the characteristic NTP-binding motifs and thus are predicted to be the putative viral replication-associated protein (RAP) genes. The remaining 4 DNA components are unrelated to each other or to segment 2, 5 and 6, based on their distinctive deduced amino acid sequence. The SCSV DNAs have no significant nucleotide sequence homology with the genomes of geminiviruses although some homology exists at the deduced amino acid level.

The replicative competency of SCSV has been demonstrated (Chu *et al.*, 1993b). Since the SCSV virion DNA is single-stranded and the transcripts are of viral sense, the first likely biosynthetic event after uncoating is likely to be the synthesis of the replicative form DNA using host DNA polymerase. (The DNAs of SCSV have the ability to self prime in dsDNA synthesis [Chu and Helms, 1988]). Host RNA polymerase is thought to bind to the promoters initiating RNA transcription followed by synthesis of the viral proteins required for virus multiplication.

In a particularly preferred embodiment of the present invention, there is contemplated an SCSV promoter or promoter-like sequence comprising a nucleotide sequence selected from within SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6 and SEQ ID NO: 7 and/or genetic constructs comprising same.

A "promoter-like sequence" as used herein includes any functional mutant, derivative, part, fragment, homologue or analogue of a naturally occurring SCSV promoter. Promoter-like sequences contemplated herein include single or multiple nucleotide substitutions, deletions and/or additions to an SCSV promoter, provided that the said promoter-like sequences retain at least 35%, preferably at least 45%, more preferably at least 55%, even more preferably at least 65-70% and still more preferably at least 85-95% or greater promoter activity compared with the corresponding wild-type SCSV promoter.

In yet another embodiment, there is provided a genetic construct comprising an SCSV promoter or promoter-like sequence and which is operable in a plant cell, said promoter or promoter-like sequence corresponding to all or part of any one of SEQ ID NO: 1 to 7 or capable of hybridising under a range of stringency conditions, ranging from high to low stringency conditions to at least one of SEQ ID NO: 1 to 7. Conveniently, a mutant, derivative, part, fragment, homologue or analogue of an SCSV promoter is defined as being functional in a plant cell and capable of hybridising under a range of at least high to low stringency conditions to at least one of SEQ ID NO: 1 to 7.

In a particularly preferred embodiment, the genetic construct further comprises a termination and/or polyadenylation sequence from SCSV or from non-SCSV source and which enhances or other facilitates expression of a gene operably linked to said promoter.

For the purposes of defining the level of stringency, reference can conveniently be made to Sambrook *et al* (1989) which is herein incorporated by reference where the washing steps at pages 9.52-9.57 are considered high stringency. A low stringency is defined herein as being in 0.1-0.5% w/v SDS at 37-45°C for 2-3 hours. Depending on the

source and concentration of nucleic acid involved in the hybridisation, alternative conditions of stringency may be employed such as medium stringent conditions which are considered herein to be 0.25-0.5% w/v SDS at ≥ 45°C for 2-3 hours or high stringent conditions as disclosed by Sambrook *et al* (1989).

Another embodiment of the present invention contemplates a method of expressing a foreign gene in a plant cell, said method comprising introducing into said plant cell a genetic construct comprising and SCSV promoter or promoter-like sequence operable in said plant cell and operably linked to said foreign gene. In a further embodiment, multiple SCSV promoters are used to drive one or more transgenes without antagonism. In yet a further embodiment, the SCSV promoter is associated with the SCSV segment 2 gene in order to enhance the expression of the foreign gene. In another embodiment, the genetic constructs further comprises one or more termination and/or polyadenylation sequences which are located at the 3′ end of the foreign gene. These sequences enhance or otherwise facilitate expression of the foreign gene. The termination and/or polyadenylation segment may be from SCSV or from another source such as the *F. bidentis* MeA gene.

In still yet another embodiment, the present invention contemplates a transgenic plant carrying an SCSV promoter or promoter-like sequence as hereinbefore defined in its genome and optionally a termination and/or polyadenylation sequence to enhance expression of a gene downstream of said promoter. Preferably, the transgenic plant exhibits altered characteristics due to expression of a genetic sequence such as a gene, oligonucleotide or ribozyme downstream of the SCSV promoter.

The present invention is further described by reference to the following non-limiting figures and/or examples. Reference herein to a promoter region from SCSV is abbreviated to "S" for SCSV, the genome segment number (e.g. 1, 3, 4, 5 and 7) and "nc" for non-coding region. For example, the promoter from SCSV genome segment 1 is defined as "S1nc". Terminator sequences for particular SCSV genome segments are indicated for example, as follows: "SC1Tr" or "SC5Tr" for the terminator sequences for segments 1 and 5, respectively. Genetic constructs comprising an SCSV promoter, a

reporter gene such as *GUS* and a terminator sequence such as from SCSV is abbreviated to "SCSV:*GUS*:SCTr" or "SCSV:*GUS*:SCSVTr". Specific promoters and termination sequences are defined as above, for example S4nc:*GUS*:SC1Tr or S4nc:*GUS*:Me3", "S4nc:*GUS*:Me3". In the latter construct the terminator sequence from the MeA gene of *Flaveria bidentis* is used, referred to herein as "Me3".

In the Figures:

FIGURE 1 shows the structures and transcription units found in a representative DNA component of a typical geminivirus and SCSV, both of which contain a ssDNA genome.

FIGURE 2 shows the seven DNA segments found in the genome of SCSV in a linear form, indicating the positions of the stem-loop structure, the common region, the open reading frame (ORF), the TATA box and the termination and polyadenylation signals on each DNA.

FIGURE 3 shows the construction of the seven SCSV DNA non-coding region: β-glucuronidase (GUS) fusion expression vectors for transformation into tobacco plants. The amplified PCR fragments were separately cloned in front of the GUS gene in pHW9 at the BamHI (B) and *Nco*I (N) sites as indicated. The resultant recombinant pHW9 vectors were cut at the *Eco*RI site and cloned into the *Eco*RI site of the recipient PGA470 binary vector.

FIGURE 4 shows the construction of the segments 5 and 7 promoter:*GUS* fusion expression vectors and their deletion derivatives for protoplast studies. DNAs corresponding to the full-length non-coding regions were obtained by PCR and cloned into pKGO in front of GUS by blunt end ligation at the *Sal*I site as indicated. The deletion derivatives were obtained by digesting the pKGO clones containing the full-length sequence with HindIII or PstI on the vector and the appropriate restriction enzymes on the SCSV sequence as indicated. The deleted pKGO constructs were religated after end-filling as required.

FIGURE 5 shows the construction of the recombinant binary pTAB10 vector (pBS150) containing the seg 7 promoter(57nc):*GUS* fusion gene for transformation into subterranean clover plants. The 57nc:*GUS* expression cassette was excised from the pKGO construct (Fig. 4) by digestion with *Hin*dIII and *Bam*HI and blunt-end ligated to pTAB10 at the *Eco*RI site after end-filling the DNAs.

FIGURE 6 shows the complete sequences of the seven SCSV DNA circles. The sequence of the non-coding region on each DNA used in the construction of the expression cassettes are underlined.

FIGURE 7 shows GUS expression detected by histochemical staining on leaves of transgenic tobacco and subterranean clover transformed with the SCSV seg 7 promoter(S7nc):*GUS* fusion expression cassette.

FIGURES 8A to 8F are photographic representations of histochemical staining for GUS activity in transgenic plants - bright field. Bright field exposures of stained leaf pieces (L), stem sections (S), roots (R) and pollen (P) from tobacco plants transformed with the GUS fusion constructs containing the SCSV promoter regions from segments 1, 3, 4, 5 and 7 referred to as S1nc, S3nc, S4nc, S5nc and S7nc promoter regions and from non-transformed plants (NT). Blue colouration indicates GUS expression. Each leaf, stem or root piece represents an independent transformant (except the top two root pieces of S5nc which could not be separated easily) and the pollen samples were mixtures from two or more transformants. Fig. 8a shows differential expression of GUS in plants transformed with either S4nc or S5nc regions compared to a non-transformed plant (NT).

FIGURES 9A to 9E are photographic representations showing histochemical staining for GUS activity in transgenic plants - dark field. Transverse (T) and longitudinal(L) thin sections of stained, embedded stem pieces from tobacco plants transformed with the GUS fusion constructs containing the component 1 (S1nc), 3 (S3nc), 4 (S4nc), 5 (S5nc), and 7 (S7nc) promoter regions, viewed with a dark field. Pink crystals indicate GUS expression. The stem pieces used for the transverse and longitudinal sections represent

independent transformants. The magnifications are: 375X in S1nc T and L, S3nc L, S4nc T, S5nc T and L, and S7nc L; 480X in S3nc T; 200X in S4nc L; and 300X in S7nc T.

FIGURE 10 is a graphical representation of a fluorometric assay for GUS expression. Fluorometric assay results from leaf extracts of tobacco plants transformed with the GUS fusion constructs containing the component 1 (S1nc), 3 (S3nc), 4, (S4nc), 5 (S5nc), and 7 (S7nc) promoter regions. Each column represents an independent transformant. GUS activities were measured with a Labsystems Fluoroskan at 5 or 10 minute intervals over 60 minutes (or 30 minutes for S4nc) using 4-methylumbelliferyl β-D-glucuronide (MUG) as the substrate. The rates of GUS activity are expressed as fluorometric units (Fl.U.) per minute per mg of protein. 1000 Fl.U is approximately equal to 825 pmoles of 4-methylumbelliferone (MU).

FIGURE 11 is a diagramatic representation of the SCSV segment 2 promoter (S2nc) construct capable of directing GUS expression in tobacco protoplasts. A fragment of segment 2 DNA from *Nco*I-*Xba*I was fused to the promoterless GUS vector, pKGO. Pr, promoter, seg 2, segment 2 of SCSV.

FIGURES 12a and 12b are diagramatic representations showing constructs of SCSV:*GUS*:SCSV Tr expression vectors. The termination/polyadenylation sequences for segment 3 of SCSV (SC3Tr) and segment 5 (SC5Tr) were amplified by PCR and cut with the respective restriction enzymes and then cloned into recombinant pKGO vector as indicated. The SC3Tr construct was cloned as an *Eco*RI-*Xho*I fragment into the pKGo vector containing S1nc:*GUS*:OCS3' to make S1nc:*GUS*:SC3Tr. The SSC5TR construct was made as an *Eco*RV fragment into Vector pKGO containing S4nc:Gus:OCS3' to make S4nc:*GUS*:S5Tr.

FIGURES 13A to 13D are photographic representations showing GUS expression in potato plant tissues directed by the SCSV segment 4 promoter (S4nc). A. stem; B, leaf; C. stolon; and D. tuber.

FIGURE 14 is a photographic representation of GUS expression in cotton leaf directed by the SCSV segment 7 promoter (S7nc).

FIGURE 15 is a representation of the MeA3's terminator sequence of the *Flaveria bidentis* MeA gene (Me3). The stop codon is shown in bold face type at the beginning of the sequence. This sequence wads engineered in the Chimeric construct to include an *Eco*R1 site: GAATTCGTTTAG.... The chimeric constructs thus contained a sequence begining AATTCGTTTAG.

FIGURE 16 is a diagramatic representation of GUS expression vectors (ME20 and ME29) containing the indicated *Flaveria bidentis* MeA gene regulatory elements.

FIGURE 17 is a diagramatic representation of the construction of S4nc plasmid pBS237 containing the expression cassette S4nc:*GUS*:Me3'. Plasmid pBS218 was digested with *Eco*R1 to remove OCS3' region and ligated with an *Eco*R1.

FIGURE 18 is a diagramatic representation of the construction of plasmid pBS246 containing the S1nc:nptII:SC3Tr expression cassette. The *Sa*1I-*Sa*1I fragment is approximately 8.5-9 khp; B_L is about 0.5 kbp; B_R is about 0.6 khp. SC1nc:nptII:SC3Tr is about 1.7 khp.

FIGURE 19a-c are diagramatic representations of the construction of plasmid pKHAN4 from pKHAN2 and pKSB.bar1. pKHAN4: A *Hind*III-*Eco*R1 segment containing S7nc (572 bp), nptII coding region (978 bp) and vicillin 3' end (276bp) from pKHAN2 was inserted into binary plasmid pKSB.bar1 to yield pKHAN4; pKHAN2. The nptII coding region (978bp, *Bam*HI-S*ma*1 fragment) from p35SKN was cloned into Asp718 site (blunted with klenow fragment) of pKHAN1 to create pKHAN 2. SCSV Pro = S7nc; pKSB. Bar 1: pTAB10.MCSori1B digested with *Eco*R1 as ligated together.

FIGURE 20 is a photographic representation of a selection of Kananmycin resistant tobacco plants on regeneration medium transformed with binary vectors containing either 35S promoter:NPTII:35S terminator sequence (35SPrm:NPTII:35STrm) or

S1nc:NPTII:SC3Tr expression cassette. (In the Figures, the abbreviations are 35S Prm NPTII35STrm and SC1 Prm NPTII Sc3 Trm, respectively.

FIGURE 21 is a diagramatic representation of a cloning vector utilising SCSV DNA transcription regulatory signals.

FIGURE 22 is a diagramatic representation of SCSV segment 2 dimer construct pBS2. This construct was created by cloning a tandem repeat of the SCSV segment 2 DNA (containing a whole functional seg 2 transcription unit) into the polylinker site of pGEM7 which was then cloned into a reduced version of the pMCP3 binary vector (Khan *et al.*, 1994).

A summary of the transcription activities facilitated by the SCSV transcription regulatory elements is shown in Table 19.

EXAMPLE 1

SCSV DNA sequence determination

The F isolate of SCSV (Chu et al. 1993a) was used for sequence determination. Full-length clones of the SCSV genome components were created from the replicative form (RF) DNA as described by Chu et al. (1993a). Other clones were created from the RF by PCR with SCSV specific primers designed from known sequences.

The dideoxy chain termination method (Sanger et al., 1977) was used to sequence either M13 ssDNA templates (Sambrook et al., 1989) or dsDNA templates prepared by the CTAB method (Del Sal et al., 1989) from clones described above. Sequence analysis was carried out using the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package (Devereaux et al., 1984).

EXAMPLE 2

Plasmid construction

DNA manipulation techniques used were as described by Sambrook et al. (1989). The entire non-coding regions of all 7 DNA segments of SCSV were amplified by PCR using specific primers (See Table 8) with appropriate restriction enzyme recognition sites for cloning into the plasmid vectors. The amplified DNA fragments were separately cloned into the respective expression vectors upstream of a promoterless GUS reporter gene in the appropriate orientation to produce the SCSV promoter-GUS fusion constructs as shown in the Figures 3 and 4. The expression vector pHW9 (Fig. 3) followed by cloning into the binary vector pGA470 (An et al., 1985) were used for tobacco transformation. The expression vector pKGO was used for GUS expression in protoplasts (Fig. 4) and the binary vector pTAB10 was used for subterranean clover transformation (Fig. 5). pKGO was constructed by cloning the *XhoI* fragment of pKIWI101 (Janssen and Gardner, 1989) containing the GUS gene and the OCS terminator sequence into the *SalI* site of pJKKm (Kirschman and Cramer, 1988). Corresponding plasmids containing the 35S promoter fused to GUS were used as controls. The junctions of the clones were checked by sequencing. A promoterless GUS construct was used as a control.

Deletion derivatives of the SCSV segments 5 and 7 promoters were made by digesting the full-length non-coding sequence with an appropriate enzyme to produce the desired deletion (Fig. 4).

To investigate the phenomenon of transactivation, the S5nc:*GUS* expression cassette was excised from recombinant pKGO constructs and cloned into a pGEM plasmid containing the seg 2 RAP coding region expressed from the 35S promoter. The resultant plasmid carrying both the SCSV promoter:*GUS* and the 35S promoter:seg 2 RAP expression cassettes were electroporated into protoplasts.

EXAMPLE 3

Protoplast isolation and transient gene expression

Recombinant plasmids were extracted from *E. coli* by alkaline lysis followed by purification through Qiagen columns as described in the Qiagen Plasmid Handbook.

Suspension cell cultures were used to isolate *Nicotiana plumbaginifolia* (Last *et al.* 1991) and subterranean clover cv. Woogenellup protoplasts (Chu *et al.* 1993b). Purified plasmids were electroporated into protoplasts of subterranean clover or *Nicotiana plumbaginifolia* as described by Taylor and Larkin (1988). Protoplasts were harvested 3 days later and assayed fluorometrically for transient GUS activity using 4-methyl umbelliferyl β-glucuronide as substrate (see below). All experiments were done using duplicate samples per treatment.

EXAMPLE 4

Transformation of tobacco with SCSV-promoter-GUS fusion constructs

The recombinant pGA470 binary constructs containing the various SCSV promoter:*GUS* expression cassettes were separately transformed into *Agrobacterium tumefaciens* strain LBA4404 (Hoekema *et al.* 1983) by electroporation as described by Nagel *et al.* (1990). *Nicotiana tobacum* cv. Wisconsin 38 were transformed and regenerated as described by Ellis *et al.* (1987).

EXAMPLE 5

Transformation of subterranean clover with SCSV-promoter-GUS fusion construct

The recombinant pTAB10 binary vector containing the S7nc:*GUS* fusion construct (pBS150) was transformed into *Agrobacterium tumefaciens* strain AGL1 (Lazo *et al.* 1991) by triparental mating (Ditta *et al.* 1980). Subterranean clover cv. Larisa was transformed by Agrobacterium-mediated transformation and regenerated as described by Khan *et al.* (1994).

EXAMPLE 6

GUS assays

Protoplasts were lysed by sonication in the presence of 0.3% v/v Triton X-100 immediately after harvest. GUS activity of the soluble extract was determined in a fluorometric assay using the substrate 4-methyl umbelliferyl β-D-glucuronide (MUG) (Jefferson *et al.*, 1987). Fluorescence was measured using a Labsystem Fluoroskan II spectrophotometer.

GUS expression in intact transgenic tissues was detected by histochemical staining in 5-bromo-4-chloro-3-indolyl-β-glucuronic acid (X-gluc) (Jefferson *et al.*, 1987) and by fluorometric assay of the soluble extract using MUG.

EXAMPLE 7

Sequence of SCSV non-coding region

The complete sequences of all the 7 known SCSV DNA circles have been determined (Fig. 6). Each DNA contains a non-coding region with signals (TATA boxes) for promoter activity (Table 2). These sequences have not been described or isolated before. Sequence comparison of the non-coding regions comprising these promoters showed that segments 3 and 5 are most similar, sharing 258 conserved nucleotides out of an average of 491 nucleotides in the non-coding regions of the DNAs. Segments 3, 4, 5 and 7 contain 170 conserved bases between them while only 152 bases are conserved between Segments 1, 3, 4, 5 and 7. The sequence variations in the non-coding regions of the SCSV DNAs suggest that the transcription and replication of the different SCSV genes may be regulated differently.

EXAMPLE 8

Transient activity of SCSV promoters in protoplasts

The promoter activities of two SCSV DNA non-coding regions have been demonstrated directly by transient expression of GUS using SCSV promoter:*GUS* fusion constructs in subterranean clover (Table 3) and tobacco protoplasts (Table 4). These results showed that both segment 5 and segment 7 promoters are functional in the absence of other

SCSV DNA components. The SCSV promoters are also functional in protoplasts of either a natural host (subterranean clover) or a non host (tobacco). In tobacco protoplasts, the segment 7 promoter was similar in activity to the 35S promoter while the segment 5 (coat protein) promoter consistently showed activity about half that of the CaMV 35S promoter (Table 4). However, the activity of both promoters were higher in subterranean clover protoplasts, with the activity of the segment 7 promoter showing up to several times that of the 35S promoter (Table 3), suggesting that SCSV promoters work better in certain legumes than the widely used 35S promoter. The activity of the segment 7 promoter also appeared to be more variable in subterranean clover protoplasts than the others tested.

Plasmids containing various deletion derivatives of the non-coding sequence of segments 5 and 7 fused to GUS were also constructed (Fig. 4) and electroporated into protoplasts (Table 5). GUS assays of protoplasts transfected with these constructs showed that neither the stem-loop nor the common region were necessary for promoter activity although the latter was required for full activity (Table 5). The DNA sequence required for high level promoter activity appears to be less than 300 bp which is smaller than that required for other promoters, such as 35S promoter (Odell *et al.*, 1985).

EXAMPLE 9

Transactivation of SCSV promoter activity by SCSV segment 2 gene product

When co-electroporated with a 35S promoter:seg 2 RAP gene construct, the activities of GUS driven by the seg 5 promoter apparently increased by about 2 fold in both subterranean clover and tobacco protoplasts (Table 6). The segment 7 promoter activity may also be increased when co-electroporated with the 35S:seg 2 RAP construct but further experiments are needed to confirm this.

Transactivation of GUS activity was also apparently observed when the S5nc:*GUS* construct was co-electroporated with a binary vector plasmid (pBS2) containing a tandem repeat (dimer) of the segment 2 DNA (Table 6). A map of SCSV segment 2 dimer construct -pBS2 is shown in Figure 22. This construct was created by cloning a tandem repeat of the SCSV segment 2 DNA (containing a whole functional seg 2

transcription unit) into the polylinker site of pGEM7 which was then cloned into a reduced version of the pMCP3 binary vector (Khan *et al.*, 1994). The results suggest that both SCSV promoters are expressed concurrently. Thus, different SCSV promoters can either be used in combination with a 35S promoter or be used simultaneously to facilitate concurrent expression of multiple transgenes in plants. In contrast, reduced transgene activity has been observed when the 35S promoter is used in multiple or multiple copy transgenes (Linn *et al.*, 1990; Matzke and Matzke, 1991; Scheid *et al.*, 1991; Carvalho *et al.*, 1992).

EXAMPLE 10

Activity of SCSV promoters in transgenic plants

All the SCSV promoters, except those from segments 2 and 6, have been shown to be capable of driving expression of transgenes in tobacco plants (Fig. 7). The level of activity of the promoters measured by the MUG assay varied from plant to plant and from one promoter to another but is generally lower than that of the 35S promoter (Table 7).

Histochemical staining of intact transgenic tobacco tissues showed that the activity of the S4nc promoter appears to be constitutive and GUS activity was detected in all plant organs. The others are generally restricted to the vascular tissues although expression is also detected in pollen.

In transgenic subterranean clover plants expressing the S7nc:*GUS* gene construct, histochemical staining showed that GUS activity is found in leaves, stems and petioles. The distribution of GUS activity is mostly constitutive although in some tissues, the activity is largely in vascular tissues (Figure 7). In these plants, the level of promoter activity also varies from plant to plant but the activity is generally comparable to that of the 35S promoter.

These results indicate that the SCSV promoters provide a choice of promoters that can be used either independently or simultaneously to control the expression of one or more foreign genes in a wide range of plants and tissue types. Legumes are a major target

for application. The activity of these promoters also can be enhanced by the presence of the seg 2 gene product. Thus, these promoters appear to have significant advantages over the CaMV 35S promoter, both in levels of expression, size and in overcoming some negative features of the 35S promoter. They would be applicable in a wide range of transgenic applications.

TABLE 1

Properties of plant viruses with small and multiple encapsidated circular ssDNAs (Plant Circoviruses)

Virus	Hosts	Vector	No. of circles	Size (kb)	Coat protein (kDa)
FBNYV	Legumes	Aphids	2+	1	22
MDV	Legumes	Aphids	1+	1	21
SCSV	Legumes	Aphids	7	1	19
BBTV	Monocots	Aphids	6+	1-1.2	20
CFDV	Monocots	Planthopper	1+	1.3	25

Compiled from data presented at the Sixth International Congress of Plant Pathology, Montreal, July, 1993. FBNYV = faba bean necrotic yellow virus, MDV = milk vetch dwarf virus, SCSV = subterranean clover stunt virus, BBTV = banana bunchy top virus, CFDV = coconut foliar decay virus.

TABLE 2

Transcription units and putative gene functions of the SCSV DNA segments

DNA Segment	Segment Size (bases)	Region Function	Non-Coding (bases)	TATA Box Pos[a]	TATA Box Sequence	ORF Pos[b]	ORF # aa	ORF Mr (kDa)	Termination Codon	Polyadenylation Signal
1	1001	Unknown	661	201	TATAAAT	48	112	12.7	TGA	AATTAT
2	1022	Replication	178	-8	TATATAT	102	280	32.5	TGA	AATAAA
3	991	Unknown	495	326	TATAAAT	57	164	19.1	TGA	AATAAA
4	1002	Unknown	539	285	TATAAAT	71	153	17.7	TAA	AATAAA
5	998	Coat protein	487	330	TATAAAT	48	169	18.7	TAA	AATAAA
6	1017	Replication	158	-7	AATATAA	66	285	33.5	TGA	AATAAA
7	988	Unknown	546	324	TATAAAT	67	146	16.9	TAA	AATAAA

[a] Position of the TATA box is the number of bases from the first nucleotide of the stem-loop to and including the first base of the TATA box. See Fig. 1 for a graphic presentation of the genome segments.

[b] Position of the ORF is the number of bases from the first base of the TATA box to and including the first base of the initiation codon.

TABLE 3

SCSV promoter directed GUS activity in subterranean clover protoplasts

Treatments	GUS Activities				
	Expt 1		Expt 2		Average
	Act	%35S	Act	%35S	%35S
No DNA	10	0%	13.1	0%	0%
35S:*GUS*	27.3	100%	36.6	100%	100%
S5nc:*GUS*	22.6	73%	40.2	116%	95%
S7nc:*GUS*	36	147%	117	385%	266%

All experiments were done using duplicate samples per treatment. GUS activity was measured using a Labsystem Fluoroskan II spectrophotometer and is presented both in absolute activity (Act) and as a percentage of 35S:*GUS* activity (%35S).

' constructs are represented as "promoter:reporter gene". For example, "35S:*GUS*" is the 35S promoter and the GUS reporter gene. "S5nc" and "S7nc" are the promoters for segments 5 and 7, respectively of SCSV.

TABLE 4

SCSV promoter directed GUS activity in tobacco protoplasts

Treatments	GUS Activities				
	Expt 1		Expt 2		Average
	Act	%35S	Act	%35S	%35S
No DNA	13.8	0%	10.5	0%	0%
35S:*GUS*	113	100%	117	100%	100%
S5nc:*GUS*	52.3	39%	61	48%	44%
S7nc:*GUS*	93	80%	106	90%	85%

All experiments were done using duplicate samples per treatment. GUS activity was measured using a Labsystem Fluoroskan II spectrophotometer and is presented both in absolute activity (Act) and as a percentage of 35S:*GUS* activity (%35S).

TABLE 5

Promoter activities (GUS expression levels) of deletion derivatives of segment 5 and 7 non-coding regions in protoplasts. Levels are expressed as percentages of the activity of the respective full-length non-coding sequence.

		GUS Activity			
		Tobacco		Subclover	
Promoter	Deletion[a]	Expt 1	Expt 2	Expt 1	Expt 2
Segment 5	dNde	101	110	-	-
	dPml (stem-loop)	65	88	-	-
	dAfl (Stem-loop+ common region)	70	102	63	91
	dPst	5	18	6	4
Segment 7	dAfl (stem-loop+ common region)	50	55	67	39

[a] See Figure 4 for maps of deletion derivatives.

TABLE 6

Transactivation of segment 5 promoter activity (GUS expression) in tobacco and subterranean clover protoplasts by gene product of segment 2.

Promoter Construct	Protoplasts	GUS Activity[a] Expt 1	Expt 2	Expt 3	Expt 4
S5nc:*GUS*	Tobacco	100	100	-	-
S5nc:*GUS* + 35S:Seg 2 RAP	Tobacco	262	217	-	-
S5nc:*GUS*	Subclover	100	100	100	100
S5nc:*GUS* + 35S:Seg 2 RAP	Subclover	132	176	188	770
S5nc:*GUS* + Seg 2 dimer[b]	Subclover	-	-	-	320

[a] GUS activities are expressed as percentages of the S5nc:*GUS* construct in each experiment

[b] See Example 9

TABLE 7

Relative GUS activity in leaf extracts of independent transgenic tobacco plants containing different promoter:*GUS* constructs as determined by fluorometric assays[a]

Promoter		GUS Activity	Average
Non transgenic control		123	
	Average		123
35S	#3	38,431	
	#6	20,052	
	#8	20,648	
	#11	28.325	
	#12	24,700	
	Average		26,431
S4nc	#1	7,229	
	#2	4,690	
	#5B	4,300	
	#9	6,218	
	#18	4,895	
	#19	6,098	
	Average		5,572
S7nc	#4	642	
	Average		642

[a] GUS activity presented is the best 25% of the transgenic plants tested.

EXAMPLE 11

Further characterisation of SCSV promoter activities in transgenic plants

Transgenic plants of tobacco transformed with the five (Segments 1, 3, 4, 5 and 7) SCSV promoter:*GUS* fusion cassettes were assayed for GUS activity by both histochemical (Figs. 8 and 9) and fluorometric assays (Fig. 10). Samples taken from tissue-cultured and young glasshouse-grown plants produced the same GUS expression pattern. GUS activity was observed in all plant parts, including roots, stems, leaves, petioles and all flower parts. Promoter 5 construct gave relative lower GUS expression in pollen than other promoters. The results from fluorometric assays confirmed previous data showing that segment 4 promoter was the highest expressor, with activity 10-fold or greater than the rest (Fig. 10), but is still lower than that of the 35S promoter. The expression levels of the segment 1, 3, 5 and 7 promoters were comparable to those of the phloem-specific promoter rolC in tobacco (Schmulling *et al.*, 1989; Sugaya *et al.*, 1989). Plants transformed with the promoterless GUS construct did not express GUS by either assay method. Histochemical assays showed that expression of all promoter constructs was the highest in vascular tissues, with high expressors being more constitutive than low expressors which are more vascular-limited (Fig. 8). In general, promoter 1, 3, 5 and 7 constructs are expressed mostly in the vascular tissues. In particular, GUS expression by promoter 1 and 3 constructs are mainly restricted to phloem tissues. However, for all promoters histochemical staining of leaves showed that GUS expression in these tissues are often blotchy (constitutive and vascular-limited) and variable between leaves of the same plant. Dark field microscopy (Jacobsen-Lyon *et al.*, 1995) also showed that none are strictly vascular-limited (Fig. 9).

Twenty primary transgenic subterranean clover plants expressing the seg 7 promoter:*GUS* gene were further characterised by histochemical assays (Table 9). These assays showed that GUS activity was observed in all plant parts, including roots, stems, leaves and petioles. GUS expression was the highest in vascular tissues, with some leaves being more constitutive and blotchy than other organs and high GUS

expressing plants being more constitutive than low expressing ones. Samples taken from tissue-cultured and glasshouse-grown plants produced the same GUS expression pattern.

EXAMPLE 12

Detection of promoter activity in SCSV segment 2 DNA

Experiments showed that the non-coding regions from the SCSV segments 2 and 6 DNAs were unable to drive the expression of GUS gene in transgenic tobacco. These regions are only 179 and 159 nucleotides long, respectively, and it is likely that additional sequences are required for promoter activity. To test this hypothesis, a new segment 2 promoter sequence was constructed consisting of the DNA fragment from nucleotides 526 to 46 and fused to the promoterless GUS vector pKGO (Fig. 11). The fusion construct was electroporated into tobacco protoplasts. Gus activity was detected in electroporated tobacco protoplasts at levels similar to that of segment 5 promoter:*GUS* construct (Table 10).

A binary vector containing the above SRnc promoter:*GUS* fusion DNA was also transformed into tobacco plants as described in Example 4. Histochemical staining of several transformed tobacco plants showed that GUS expression was mainly vascular. These results showed that additional sequence from the SCSV segment 2 DNA coding region is necessary for promoter function. Since the SCSV segment 6 is a variant of the segment 2, it is expected that a similar construct comprising the noncoding and part of the coding region of this DNA will produce an active promoter. Thus, all SCSV promoters are expected to be suitable for driving gene expression in plants.

EXAMPLE 13

Enhancement of gene expression by SCSV transcription termination and polyadenylation signals

Effective gene expression requires not only a promoter, but also specific nucleotide sequences at the 3' end of the coding region of the gene, known as the termination and polyadenylation signals (Messing *et al.*, 1983; Joshi 1987b; Gil and Proudfoot, 1984; Rothnie *et al.*, 1994). These special sequences are required to signal the RNA polymerase to stop transcription and to allow further processing of the RNA transcript. The activity of the termination and polyadenylation signals may affect the transcription efficiency and stability of the RNA being transcribed. Some widely used termination/polyadenylation sequences include those from the NOS (Depicker *et al.*, 1982), OCS (Janssen and Gardner, 1989) and CaMV 35S (Pietrzak *et al.*, 1986) genes.

Each of the SCSV DNA segment (or component) contained a terminator sequence comprising a termination and a polyadenylation signal sequence in the noncoding region (Table 11; Figure 2). To demonstrate activity of the termination/polyadenylation signals in SCSV DNA, GUS expression vectors containing either the segment 3 or segment 5 polyadenylation and termination signal (Fig. 12) was constructed and subjected to transient expression in tobacco protoplasts. The respective terminator sequence was amplified by PCR with several restriction sites incorporated into the primers (Fig. 12). The amplified terminator fragments were cut with the indicated restriction enzymes and cloned into the pKGO recombinant plasmid containing either S1nc or S4nc promoter:*GUS*:OCS3' constructs, from which the OCS3' sequence has been deleted (Fig. 12). The resultant S1nc:*GUS*:SC3Tr (the segment 1 promoter here carries a deletion of the *Hind*III fragment from nucleotides 641-782 which has no effect on GUS activity) and S4nc:*GUS*:S5Tr constructs were electroporated into tobacco protoplasts and assayed for GUS activity. The results showed that GUS activity was increased 2 to 3-fold when the SCSV termination/polyadenylation sequence was used instead of the commonly used OCS termination/polyadenylation sequence in the same construct (Table 12). In the same

experiment, the construct S1nc:*GUS*:S3Tr produced over two-fold higher activity than the 35S:*GUS*:OCS3' construct (Table 12). These results indicate that each of the SCSV DNA components contains a different termination and polyadenylation signal sequence which can be used in various combination with the SCSV promoters to regulate and/or enhance expression of foreign genes in plants. As with the SCSV promoter sequences, the SCSV termination/polyadenylation sequences are advantageous over currently available termination/polyadenylation sequences by their small sizes (160-170 nucleotides) and the availability of a broad range of transcription regulators with different strengths and tissue specificities for genetic manipulation. The results also show that the use of a SCSV promoter in combination with a SCSV terminator sequence in higher levels of gene expression than constructs using the 35S promoter in conjunction with one of the common transcription terminator sequences.

EXAMPLE 14

Activity of SCSV promoters in transgenic potato plants

pGA470 binary vector containing the S4nc:*GUS*:NOS fusion construct cloned in pHW9 (Figure 3) was used to transform potato plants. pHW9 is derived from pHW8 (Dolferus *et al.*, 1994) into which the polylinker from pGEM3zf(+) is inserted. The recombinant binary vector was transformed into *Agrobacterium tumefaciens* strain LBA4404 by electroporation (Nagel *et al.*, 1990). Potato cultivars Atlantic and Sebago were transformed and regenerated essentially as described by Wenzler *et al.* (1989) except for the following modifications. Stem pieces instead of leaf pieces were used for transformation and 10 mg/l of benzylaminopurine (BAP) instead of 2.24 mg/l was used during co-cultivation. After co-cultivation, stage 1 medium is supplemented with 100mg/l of cefotaxime and not kanamycin or carbenicillin. Stage II medium contained 2 mg/l BAP, 5 mg/l GA3, 100 mg/l kanamycin and 100 mg/l cefotaxime.

Six transformed plants comprising 5 of cultivar Atlantic and 1 of cultivar Sebago were transferred and grown in the glasshouse for 10-11 weeks until small tubers formed. Tissues from different parts of the plants were assayed by histochemical GUS staining. The results showed that GUS was highly expressed in all plant parts including roots,

stolons, tubers, stems and leaves but the expression was predominantly vascular, including cambium, phloem elements and some xylem elements (Fig. 13). As in other transgenic hosts, GUS expression in non-vascular tissues of highly GUS active plant materials, especially young tubers, was more evident than in less active tissues when compared with plants transformed with a 35S:*GUS*:NOS construct, SCSV promoter directed *GUS* expression in tubers was at least as high as that of the 35S:*GUS* construct.

EXAMPLE 15

Activity of SCSV segment 7 promoter in transgenic cotton plants

pGA470 binary vector containing S7nc:*GUS*:NOS fusion construct cloned in pHW9 was used to transform cotton plants. The recombinant binary vector was transformed into *Agrobacterium tumefaciens* strain AGL1 by triparental mating. Cotton (*Gossypium hirsutum*) cv. coker 315 was transformed and regenerated as described by Cousins *et al.* (1991).

Transformed plants were grown in the glasshouse and leaf tissues from 18 independent transgenic plants were assayed for GUS activity by histochemical staining. GUS activity varied between plants. Five of these plants showed strong GUS expression, similar in range to 35S promoter driven GUS expression and was predominantly in the vascular tissues (Fig. 14). GUS activity was especially strong in the gossypol glands. As in other transgenic hosts, GUS staining in highly expressed tissues were also constitutive.

A variety of tissues from these plants were then stained and vascular expression was observed in all organs including roots, stems, petioles, petals and other vascularised floral parts. Expression appears to be particularly high in young flower buds. Seedlings from one of the lines was screened for GUS activity. All 10 progenies stained heavily in roots and leaves indicating that the gene was inherited and that the line probably contained more than one independent insertion site.

EXAMPLE 16

Stability of transformed SCSV promoter:*GUS* expression cassette in transgenic plants

The stability of GUS expression driven by the various SCSV promoters in transgenic tobacco and subclover were further characterised in T1 generation seedlings of subterranean clover and tobacco plants.

In tobacco, T1 seedlings from 10 independent transgenic lines were assayed by histochemical staining. The results showed that the expression of the GUS gene driven by all the five (segments 1,3,4,5 and 7) promoters was stable in the T1 seedlings, with the pattern of expression being maintained in all cases between T0 and T1 plant tissues of the same age. In very young stems where the vascular tissues are not well differentiated, expression from all promoters were very high and was detected through out the stem tissues. Gradual vascular limitation occurs with age and with increasing differentiation of the vascular bundles. As with T0 plants, the segment 4 promoter mediated GUS expression was more constitutive than others.

Fifty 2-3 month old T1 seedlings from 15 independent transgenic subclover plants expressing the S7nc:*GUS* fusion construct were assayed for GUS activity by histochemical and fluorometric assays. The results showed that the expression of the GUS gene driven by the segment 7 promoter was stable in T1 seedlings, with the pattern of expression being maintained between T0 and T1 plant tissues of the same age. GUS expression was found to generally segregate at the expected ratio of 3:1. The preliminary results from fluorometric assays confirmed the histochemical data suggesting that this segment 7 promoter construct had GUS activity somewhat lower than that of the 35S promoter in leaves and petioles (Table 13). GUS activity in subterranean clover stems, however, was 3-fold higher than in petioles and 6-fold higher than in leaves (Table 14). The age of the plants at the time of assay was 2 to 3 months.

EXAMPLE 17

Transient activity of SCSV promoter in soybean leaves

The SCSV promoter:*GUS* construct used (Table 15) was derived from the promoterless GUS plasmid pKGO (Figure 4; pJKKmf(-) K1W1 GUS:OCS) described previously while the 35S promoter:*GUS* construct was pGUS. pGUS is derived by cloning the *Gus* gene from pKIWI101 into the plant expression vector pDH51 (Pietrzak *et al.*, 1986).

For transient GUS expression in soybean tissues, the GUS constructs were introduced into tissues tissues by particle bombardment using the Bio-Rad Biolistic PDS-1000/He Particle Delivery System as above. A 50µl suspension containing 3mg of a 1:1 ratio of 1 and 5µ gold particles plus 6ug of DNA was shot onto plates containing 3 leaves each. Fully expanded leaves used in these experiments were prepared from 24 day old soybean plants cv. Wayne. After particle bombardment, GUS activity was assayed 24 hours later by vaccuum infiltration of the leaves with X-Gluc (Craig, 1992).

The results (Table 15) showed that in transient expression in soybean leaves, SCSV segment 4 promoter was more active (25-35 spots/leaf) than the 35S promoter (10-15 spots/leaf) when the respective plasmids were shot into soybean leaves.

EXAMPLE 18

Testing of SCSV promoters for callus-specific expression

All seven SCSV non-coding sequences were cloned into the promoterless GUS vector pHW9. Binary vectors each containing one of the seven SCSV promoter:*GUS* fusion constructs were transformed into tobacco tissues by *Agrobacterium*-mediated gene transfer. At 2-3 weeks after transformation, calli containing primodia of transformed shoots were subjected to histochemical GUS staining. Best expression was observed in calli transformed with segment 1 followed by the S4nc:*GUS*:OCS constructs. This result suggests that the segment 1 promoter is best suitable for selectable marker gene

expression and that the segment 4 promoter is best for gene expression.

EXAMPLE 19

Characterisation of regulatory sequences of the *Flaveria bidentis* MeA gene

In *Flaveria bidentis* (Chitty *et al.*, 1994) the MeA gene is the gene that codes for the NADP-malic enzyme adapted for C4 photosynthesis. The structure and putative promoter and transcription termination/polyadenylation signal sequences of this gene has been isolated and the terminator sequence determined (Fig. 15). The potential activities of the putative promoter element (MeA 5′ sequences [MeA 5′] and the terminator MeA 3′ sequences [MeA 3′]) of the *F. bidentis* MeA gene were studied in transgenic *F. bidentis* plants using GUS expression vectors (Fig. 16). A long version of the MeA 3′ terminator sequence (MeA 3′L = 5.5kb from the stop codon) was used in these experiments. In Fig. 16, the GUS expression cassette ME20 is ligated to the binary vector pGA470 (An *et al.*, 1985) while ME29 is cloned into pGA482 (An, 1986). Plants were transformed with these vectors as described by Chitty *et al.* (1994)

Study of GUS activity of the transformed plants by histochemical staining and fluorometric assays showed that the MeA 3′ sequence of the gene is required for high level expression of GUS in leaves of transgenic *F. bidentis* plants (Table 16). The 5′ sequences of the gene do not appear to contribute to gene expression in leaves but appear to direct expression in meristems and stems in the presence of a suitable transcription termination/polyadenylation signal sequence such as the OCS 3′ (Table 16).

EXAMPLE 20

Use of the MeA 3′ termination/polyadenylation signal sequences in SCSV promoter constructs to enhance gene expression in monocotyledenous plants

Because most gene control elements are located at the 5′ end, the activity of the MeA 3′ sequence is tested in conjunction with the S4nc SCSV promoter with the view to enhance gene expression directed by the SCSV promoters. For these experiments, a

short version of the MeA 3' terminator sequence was used (MeA 3'S; 900 bases from the stop codon) to prepare GUS expression vectors containing the S4nc promoter with either the OCS 3', SCSV segment 5 3' (SC5Tr) or the MeA 3' transcription termination/polyadenylation signal sequence. These constructs were derived from the promoterless GUS plasmid pKGO described previously and the recombinant plasmid pBS237 containing the S4nc:*GUS*:MeA3' construct is presented in Fig. 17. GUS activity conferred by these constructs were assayed in Japonica rice callus cv. Taipei 309. The constructs were introduced into rice calli by particle bombardment using the Bio-Rad Biolistic PDS-1000/He Particle Delivery System (Bio-Rad Laboratories) and compared with results obtained in dicotyledonous tissues such as soybean leaves and tobacco protoplasts (Table 17). For rice particle bombardment experiments, 4 mg of a 1:1 ratio of 1 and 5 micron gold particles plus 5 ug of DNA in a total of 50ul volume was shot onto six plates of calli. The DNA was made up with a 4:1 molar ratio of each of the vectors containing GUS gene to the vector containing the selectable marker gene. The vector containing the selectable marker was pTRA151 (Zheng *et al.*, 1991). Each plate contained 50-100 fresh secondary calli derived from mature embryos. Forty hours after DNA bombardment, GUS activity was detected by placing the calli in 0.3% w/v X-Gluc solution in 100 mM phosphate buffer. Blue spots were counted after overnight incubation.

For transient GUS expression in tobacco and soybean, only the GUS constructs were introduced into protoplasts and leaf tissues, respectively. After electroporation, tobacco protoplasts were assayed for GUS activity as previously described. The constructs were introduced into soybean tissues by particle bombardment as described above.

The results showed that in the monocotyledenous rice tissues, a 16-fold higher activity was obtained with the MeA 3' construct compared to the SCSV terminator (Table 17). In similar experiments, a highly expressed GUS construct containing the ubiquitin promoter:*GUS*:NOS cassette (Christensen *et al.*, 1992) has about 4-fold higher activity than the SC4:*GUS*:MeA3' construct.

In the dicotyledonous tissues, similar activities were obtained with both of these constructs in tobacco protoplasts and soybean leaves. However, both were 2-fold higher in activity than that obtained with the OCS terminator in tobacco protoplasts (Table 17).

These results suggest that the MeA3' sequence can be used to enable gene expression directed by SCSV promoters in monocots.

EXAMPLE 21

Use of new vectors containing SCSV promoters and terminators to drive a selectable marker gene in transgenic plants

The suitability of using SCSV promoters to drive a selectable marker gene as a basis for selecting transgenic plants after transformation and regeneration was tested in tobacco plants. The selectable marker used is the kanamycin resistance gene, *npt*II. Binary vectors containing either a SCSV segment 1 (pBS246) (Fig. 18) or a SCSV seg 7 promoter (pKHAN4) (Fig. 19) fused to the *npt*II gene were constructed from the pART27 (Gleave, 1992) and pKSB.bar1 (Figure 19), respectively. These were transformed separately into tobacco plants (Ellis *et al.*, 1987) and putative transgenic plants were selected under kanamycin selection using 100 µg/ml kanamycin (Fig. 20). Kanamycin resistance was confirmed in the transgenic plants by dot blot assay for the nptII gene activity (McDonnell *et al.*, 1987) and survival of the transgenic plants under 100µg/ml kanamycin in a rooting medium. The results showed that the SCSV segment promoter construct produced at least as many kanamycin resistant plants as the 35S promoter construct use in the same experiment and is, therefore, as effective as the 35S promoter for selecting transgenic tobacco plants based on kanamycin resistance (Table 18). Tobacco transformed with pKHAN4 is resistant to 100 µg/ml kanamycin in regeneration medium and 50 µg/ml kanamycin in rooting medium. Restriction maps of pKSB.bar1 and pKHAN2 used to produce pKHAN4 are shown in Figure 19.

EXAMPLE 22

Development of a new plant gene expression vector system

A new expression vector comprising a SCSV segment 4 promoter and a SCSV segment 5 terminator driving any useful gene of interest (pICAN 1) (Fig. 21) has been constructed from a pGEM derivative and the resultant expression cassette can be inserted into the binary vectors pBS246 and pKHAN4. The resultant binary vectors can then be used to transform plants of economic importance especially cotton, subclover, potato and white clover under kanamycin selection. Other binary vectors can be constructed from different combinations of SCSV promoters and terminators to produce a full range of binary vector system for plant gene expression.

TABLE 8

Sequences and positions of PCR primers for cloning non-coding regions and corresponding PCR fragment sizes.

Primer 1 name	Position of 5' end	Primer 1 sequence (5'-3')	Primer 2 name	Position of 5' end	Primer 2 sequence (5'-3')	Fragment size (bp)*
S1nc3	241	GGCGTGCGTCGGCCATGG CGCTATGAAATTCTGAAC	S1nc5	577	GGCGTGCGTCGGGGATCCTA TGTTGTAATTTTATATGG	665
S2nc3	79	GGCGTGCGTCGGCCATGG AAGCTTAGAGAGAGAAAG	S2nc5	924	GGCGTGCGTCGGGGATCCA ATAAAAGAATATATATTATTG	177
S3nc3	374	CTCACTATAGAACCATGGA CACAAGATTCTAAG	S3nc5	863	CTCACTAAAGGGGGATCCTG AGATGTAATTGTG	502
S4nc3	347	CTCACTATAGAACCATGGA AACGCAGAACAAAG	S4nc5	803	CTCACTAAAGGGGGATCCTA ATTGTTATTATCA	546
S5nc3	372	CTCACTATAGAACCATGGT CGTTGTAAAATGAC	S5nc5	874	CTCACTAAAGGGGGATCCTA ATTGTGATGATT	523
S6nc3	50	CTCACTATAGAACCATGGT GGGCCCAGGGAAGCGA	S6nc5	903	CTCACTAAAGGGGGATCCTG AAAACTCTGCGAA	164
S7nc3	383	CTCACTATAGAACCATGGC TTAAAACCAGAACA	S7nc5	817	CTCACTAAAGGGGGATCCTA ATTAATAGTAATTATG	554

* not including the spacer nucleotides at the 5' ends of the primers

TABLE 9

GUS activity of SCSV segment 7 promoter:*GUS* construct in primary transgenic substerranean clover plants (To)

Plant #	Basta Resistance	GUS Activity
1	R	+++
2	R	++
3	R	-
4	R	++
5	S	++
6	R	-
7	R	++++
8	R	++++
9	MR	+++
10	R	+++
11	R	+++
12	MR	-
13	MR	++
14	S	++
15	R	+
16	N.d.	++++
17	N.d.	+++
18	N.d.	-
19	N.d.	+++
20	N.d.	++

Plants were analysed two months after transfer of plants to glasshouse from tissue culture. Basta (phosphinothricin - [PPT] resistance was assayed by painting basta at 1 gm PPT/litre onto fully unfolded young leaves and reaction was assayed after one week.

R No damage;
MR Moderate damage to leaflet;
S Leaflet dead
N.d. Not determined

TABLE 10

GUS activity in tobacco protoplasts directed by SCSV segment 2 promoter to tobacco protoplasts relative to the 35S promoter

Construct	GUS Activity	
	Experiment	
	1	2
S2nc:*GUS*:OCS3'	0.46	0.45
35S:*GUS*:OCS3'	1	1

TABLE 11

Putative Polyadenylation and Termination signals in SCSV DNA Components

DNA Component	Putative Polyadenylation/Termination Signals
Seg. 1	AATTAT ___19___ TGTGTTTT
Seg. 2	AATAAA ___10___ TTGTTTT
Seg. 3	AATAAA ___3___ TTGTT
Seg. 4	AATAAA ___8___ TTATTGTT
Seg. 5	AATAAA ___3___ TTGTTTT
Seg. 6	AATAAA ___9___ TTGTT
Seg. 7	AATAAA ___11___ TTGTTT

TABLE 12

GUS Activity Directed by SCSV Promoter in the presence of different terminators in tobacco protoplasts

	GUS ACTIVITY			
	Expt 1	**Expt 2**	**Expt 3**	**Av**
S4nc:*GUS*:OCS3'	551	166.3	-	358
S4nc:*GUS*:SC5Tr	1258	311.8	-	785
No DNA	0	0	0	0
S1nc:*GUS*:OCS3'	-	-	26	26
S1nc[Δ*Hin*dIII][a]:*GUS*:OCS3'	-	-	29	29
S1nc[Δ*Hin*dIII]:*GUS*:SC3Tr	-	-	84	84
35S:*GUS*:OCS3'	-	-	35	35

In each experiment, duplicate electroporations of each construct was performed. Results are the average of the duplicates.

[a] Contains a deletion of a *Hin*dIII fragment.

TABLE 13

Fluorometric GUS Assay of Independent T1 generation of transgenic subterranean clover plants expressing either the S7nc:GUS or the 35S:*GUS* Construct

Construct/Plant #	Leaf (Young) [unfolded]	Petiole (Young) [unfolded]
S7nc:GUS Plant # 1	56.6	120
S7nc:GUS Plant # 2	35	48
S7nc:GUS Plant # 3	116	140
355:GUS Plant # 1	120	238
355:GUS Plant # 2	280	238

Plants were 2 - 3 months old when assayed.

Results show differential expression in different tissues.

TABLE 14

Distribution of GUS activity in a T1 generation transgenic subterranean clover plant expressing the S7nc:GUS construct

Source of Tissue	Leaf	Petiole	Stem
Top - [folded leaf; #1 leaf position]	1	1.9	2.0
Middle [#8 leaf position]	1.6	5.8	22.0
Bottom [#17 leaf position]	4.0	4.8	12.6

Plants were 2 - 3 months old when assayed.

Results show differential expression in different tissues.

TABLE 15

Transient expression of GUS in soybean leaves directed by a SCSV promoter

Constructs	GUS Expression in soybean leaves * (# spots/leaf)
S4nc:*GUS*:SC5Tr	25 - 35
35S:*GUS*:35STr	10 - 15

* Results from one experiment in which spots from 3 leaves were counted in each treatment

TABLE 16

Characterisation of GUS activity directed by MeA 3'L sequence (5.5kb version) in Transgenic *Flaveria bidentis* Plants

Constructs	GUS Expression
MeA:*GUS*:MeA 3'L (ME24)	High GUS in leaves
	High GUS in meristem
	Moderate GUS in stems
MeA:*GUS*:OCS 3'(ME20)	No GUS in leaves
	High GUS in meristem
	Moderate GUS in stems

TABLE 17

Transient expression of GUS in rice callus, soybean leaves and tobacco protoplasts showing enhancement of SCSV promoter activity by MeA 3's sequences in rice callus

Constructs	GUS Expression		
	Rice callus (Rel # spots)	Soybean leaves (Rel # spots)*	Tobacco protoplasts (Fluorescence)
S4nc:*GUS*:OCS 3'	ND[a]	ND	0.49
S4nc:*GUS*:SC5Tr	0.06	1	1.0
S4nc:*GUS*:MeA 3'S	1	1	1.1

ND Not done

* Average number of spots from 3 leaves in one experiment

TABLE 18

Selection of putative transgenic tobacco plants transformed with Kanamycin resistance gene driven by 355 or S4nc promoter.

Selection Criteria	Constructs	% Kanamycin resistant	
npt II dot Blot	35S:npt II:35STr	(1/3)*	33%
	S1nc:npt II:SC3Tr	(7/16)	43%
Kanamycin selection in rooting medium	35S:npt II:35STr	(10/21)	48%
	S1nc:npt II:SC3Tr	(11/21)	52%

* Number of resistant plants in total number of plants tested.

TABLE 19
Summary of the activities of SCSV transcription regulatory elements in plant tissues

A.	SCSV promoter	Gene Expressed	Activity Detected: Protoplasts	Activity Detected: Transgenic Plants
	Seg 1	*GUS*, *npt*II	Tobacco	Tobacco
	Seg 2	*GUS*	Tobacco	Tobacco
	Seg 3	*GUS*	Not done	Tobacco
	Seg 4	*GUS*	Tobacco	Tobacco, potato
	Seg 5	*GUS*	Tobacco, subclover	Tobacco
	Seg 6	None	Not done	Not done*
	Seg 7	*GUS*, *npt*II	Tobacco, subclover	Tobacco, subclover, cotton

B.	SCSV Terminator	Gene Expressed	Activity Detected: Protoplasts	Activity Detected: Transgenic Plants
	SC3Tr	*GUS*, *npt*II	Tobacco	Tobacco
	SC5Tr	*GUS*	Tobacco	Not done

* Sequence used probably insufficient to be active. Additional DNA sequence may be required as shown in segment 2.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION

(ii) TITLE OF INVENTION: NOVEL PLANT PROMOTERS AND USES THEREFOR

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(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1001 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TAGTATTACC CCCGTGCCGG GATCAGAGAC ATTTGACCAA TAGTTGACTA GTATAATAGC 60

CCTTGGATTA AATGACACGT GGACGCTCAG GATCTGTGAT GCTAGTGAAG CGCTTAAGCT 120

GAACGAATCT GACGGAAGAG CGTTCACACT TAGATCTAGT TAGCGTACTT AGTACGCGTT 180

GTCTTGGGTC TATAAATAGA GTGCTTCTGA ACAGATTGTT CAGAATTTCA TAGCGAGATG 240

GATTCTGGTG ATGGTTACAA TACATACTCA TATGAAGAAG GTGCTGGAGA TGCGAAGAAG 300

GAAGTTTTAT ATAAAATAGG TATTATTATG TTATGTATTG TAGGGATTGT AGTTTTATGG 360

GTTTTAATTA TATTATGTTG TGCTGTTCCT CGCTATGCTA AATCAACGAT GGACGCTTGG 420

TTATCTTCGT CTTCTATTAT GAAGAGGAAG ATGGCTTCAA GGATTACTGG TACTCCGTTT 480

GAAGAAACTG GTCCTCATCG TGAAAGAAGA TGGGCTGAAA GAAGAACTGA AGCGACGAAC 540

CAGAATAATA ATGATAATGT AAATAGATTT AGTTGATATG TTGTAATTTT ATATGGATTA 600

ATGAGAATTA TTATTATTCT GTTCTTCGTC TGTGTTTTTT AAGCTTTTTC TGTGTTTTAA 660

TGGCGTCTGG AGAGAGAAAG GAATAATTGT AAGGTAGACG ACGATGTAGT GGATTACAGT 720

TGTCTTTACT TCGCCTCGAA GAAAGACACA TTTCAAGTTG TGAGTGTTAT TGCTTTTGAG 780

GAAGCTTCCT CGAAGCAGCG TATAACTTTA ATTTGAATTT GGTTTTGGCG CGTTAGTGAA 840

ATTGCGGCTG TAAACGTGTC AAGTTGTGAG TGGCTGAAAT AAGATAATAG ATATATTATT 900

ATTGTTTTAA TTTAATTCCG CGAAGCGATA TGTTAAGTGA TAAATGAAAC GAAGCGTTTT 960

GATGACGTCA TATGTCTCCG TGCCTACGTC AGCACGGGGC T 1001

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1022 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TAGTATTACC CGACCTTGCC ACACCTCCTT GGAACACTTT CTCTCTCTAG AAAGTGTGAG 60

ACTTTCTCTC TCTAAGCTTA TATGGCTAGA AGGTACTGTT TTACATTAAA TTACGCTACT 120

GAGATAGAGA GAGAAACATT CCTCTCCCTC TTCTCTCAAG ACGAATTAAA CTATTTCGTT 180

GTCGGCGACG AAACTGCAAC TACTGGACAG AAACACCTCC AGGGATTTGT ATCGTTCAAG 240

AACAAAATTC GTCTTGGTGG ATTGAAGAAG AAATTTGGTA ATCGAGCTCA CTGGGAAATT 300

GCGAGAGGCA GCGATTCTCA GAATCGCGAT TATTGCTGTA AAGAAACCCT AATTTCTGAA 360

ATTGGGATTC CGGTCATGAA GGGTTCGAAC AAGCGGAAGA CGATGGAGAT TTATGAAGAG 420

GATCCCGAAG AAATGCAATT GAAGGATCCA GATACTGCTC TTCGATGTAA GGCGAAGAAA 480

TTGAAAGAGG AATATTGTTC CTGTTATGAT TTTCAGAAAC TCCGTCCATG GCAAATTGAG 540

CTTCACGAGG ATTTAATGGC GGAACCAGAT GATCGGAGTA TCATCTGGGT CTATGGTTCA 600

GACGGAGGAG AAGGAAAGAC GAGCTTCGCG AAGGAATTAA TCAGGTATGG ATGGTTTTAT 660

ACAGCCGGAG GGAAGACCCA GGACGTATTA TATATGTATG CTCAAGACCC AGAGAGGAAT 720

ATTGCGTTTG ATGTTCCCAG GTGTTCTTCG GAGATGATGA ACTATCAGGC GATGGAGATG 780

TTGAAGAACA GAGTTTTTGC AAGTACAAAA TATAGGCCTG TAGATCTTTG TATTAGGAAG 840

TTAGTTCATT TAATTGTGTT TGCCAACGTG GCACCTGACC CCACGCGCAT AAGTGAGGAC 900

AGACTTGTAA TTATCAATTG TTGAATAAAA GAATATATAT TATTGTTTTA ATTTAATTCC 960

GCGAAGCGGT AGCCGGTCAT AACACTGTTG CCCTTGGAAC ACTATATATA GCAAGGTCGG 1020

CT 1022

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 991 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TAGTATTACC CCCGTGCCGG GATCAGAGAC ATTTGACCAA TAGTTGACTA TGAATAATAG 60

CCCTTGGATT AGATGACACG TGGACGCTCA GGATCTGTGA TGCTAGTGAA GCGCTTAAGC 120

TGAACGAATC TGACGGAAGA GCGGACATAC GCACATGGAT TATGGCCCAC ATGTCTAAAG 180

TGTATCTCTT TACAGCTATA TTGATGTGAC GTAAGATGCT TTACTTCGCC TCGAAGTAAA 240

GTAGGAAATT GCTCGCTAAG TTATTCTTTT CTGAAAGAAA TTAATTTAAT TCTAAATTAA 300

ATTAAATGAG TGGCTATAAA TAGATGTTTC GTCTTCGTTG TTTTACAACG AAGCTTAGAA 360

TCTTGTGTTA ATGGCGTTAA GGTATTTCTC TCATCTTCCT GAAGAACTGA AGGAGAAGAT 420

TATGAACGAG CACTTGAAGG AAATTAAGAA GAAGGAATTT CTAGAGAATG TAATTAAAGC 480

TGCGTGTGCT GTGTTCGAAG GTTTAACAAA GAAGGAGTCT GTTGAAGAAG ACGACATACT 540

ACGCTTCTCT GGGTTTCTGG AAGGTCTGTC TGCATATTAT GCAGAGGCGA CGAAGAAGAA 600

GTGTTTAGTT AGATGGAAGA AGAGCGTTGC AATAAATCTG AAATGGAGAG TTATGGAGGA 660

GATGCATTAC AAGCTTTATG GATTTGCAGA CATGGAAGAT TTATATTATT CAGAGTTAGG 720

GTTTCCTAAT TACGGTGAAG ACGATGTAGC TTATCACGAT GGTGCAATTG TAAATTGTAA 780

GCAATTAGAA GTTGTATTTG ATGATTTAGG TATTGAGTTT ATGTCTATTG TAATTGATAG 840

AGGTTCTATT AAGATAGAAT TATGAGATGT AATTGTGATT AATGAATAAA GAGTTGTTAT 900

TATTCTTTGA ATTACTCCGC GAAGCGGTGT GTTATGTTTT TGTTGGAGAC ATATGACGTC 960

ATATGTCTCG CCGACAGGCT GGCACGGGGC T 991

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1002 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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GAACGAATCT GACGGAAGAG CGGACAAACG CACATGGACT ATGGCCCACT GCTTTATTAA 180

AGAAGTGAAT GACAGCTGTC TTTGCTTCAA GACGAAGTAA AGAATAGTGG AAAACGCGTA 240

AAGAATAAGC GTACTCAGTA CGCTTCGTGG CTTTATAAAT AGTGCTTCGT CTTATTCTTC 300

GTTGTATCAT CAACGAAGAA GTTAAGCTTT GTTCTGCGTT TTAATGGCGG ACTGGTTTCA 360

CAGTGCGCTT AAGACATGTA CTCATGTCTG TGATTTTTCA GATATTAAGG CGTCTTCACA 420

ACAGGATTTC TTCTGTTGTG ATAGTATGCG AGGTAAATTA TCTGAACCTA GGAAGGTGTT 480

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AGGTCAAGTT CAGTTGGGTA TGCAGCAAGA TGATGGCGTT GTTCGTCCAA TAGGATATAT 600

TCCTATTGGG GGTTATTTGT ATCATGATGA TTATGGATAT TATCAAGGAG AGAAGACGTT 660

CAATCTGGAC ATCGAGTCAG ATTATCTGAA GCCTGATGAA GATTTTTGGA AGAGATTTAC 720

AATTAATATT GTAAATGATA AAGGATTAGA TGATAGGTGT GATGTAAAAT GTTATGTAGT 780

TCATACGATG CGTATTAAGG TGTAATTGTT ATTATCAATA AAAGAATTTT TATTGTTATT 840

GTGTTATTTG GTAATTTATG CTTATAAGTA ATTCTATGAT TAATTGTGAA TTAATAAGAC 900

TAATGAGGAT AATAATTGAA TTTGATTAAA TTAACTCTGC GAAGCTATAT GTCTTTCACG 960

TGAGAGTCAC GTGATGTCTC CGCGACAGGC TGGCACGGGG CT 1002

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 998 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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GAACGAATCT GACGGAAGAG CGTCATGGTC CACATGTCTA AAGAATAATG CTTTACAGCT 180

GTATTGATTT GACTTTACGC GCTTTACTTT AATTGCTTTA AGTAAAGTAA GATGCTTTAC 240

TTTGCTCGCG ACGAAGCAAA GTGATTGTAG CTGCAGAAAT TGATGCTTTA ATTACCGGGT 300

AACACGGTTT GATTGTGGGT ATAAATATGT TCTGTTCGTT TTCTTCGTTG TCATTTTACA 360

ACGAAGATGG TTGCTGTTCG ATGGGGAAGA AAGGGTCTGA GGTCTCAAAG GAGAAAATAT 420

TCGCGAATTG CTTACAAACC TCCTTCGTCT AAGGTTGTAA GTCATGTGGA GTCTGTTCTG 480

AATAAGAGAG ATGTTACTGG AGCGGAGGTT AAGCCATTCG CTGATGGTTC AAGGTATAGT 540

ATGAAGAAGG TAATGTTGAT TGCAACATTA ACTATGGCTC CTGGAGAATT AGTTAATTAT 600

CTTATTGTGA AGAGTAATTC GCCTATTGCG AATTGGAGTT CGTCTTTCAG TAATCCTTCG 660

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TCTTCTGGTA CTGCTGGTAA AGATGTAACT AAGTCTTTTA GGAAGTTTGT TAAGCTGGGT 780

TCAGGTATTA GTCAGACCCA GCATTTGTAT TTAATTATTT ATTCCAGTGA TGCGATGAAG 840

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TGACGTCATA TGTCTCCGCG ACAGGCTGGC ACGGGGCT 998

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1017 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CAGTATTACC GCACCTCGCT TACCCTCCTC GCTTCCCTGG GCCCACTATG CCTACTAGAC 60

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CAGATTATAG AACGATAATC TGGGTGTATG GACCTGCTGG TAATGAAGGC AAATCTACAT 600

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TTAGTTCAGA GTATGTGAAT TATGGTGTAA TAGAACAGGT TAAGAATAGG GTAATGGTGA 780

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TCGGGTAGTT CGCGAAACAG GGTGAGGGAA GCGAGCAATA TAAGGCGAGG TGCGTAT 1017

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 988 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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CCTTGGATTA GATGACACGT GGACGCTCAG GATCTGTGAT GCTAGTGAAG CGCTTAAGCT 120

GAACGAATCT GACGGAAGAG CGGACATACG CACATGGATT ATGGCCCACA TGTCTAAAGT 180

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TTTTATTCGC GAAGCGGTGT GTTATGTTTT TGTTGGAGAC ATCACGTGAC TCTCACGTGA 960

TGTCTCCGCG ACAGGCTGGC ACGGGGCT 988

CLAIMS:

1. A genetic construct comprising a circovirus promoter which is operable in a plant cell.

2. A genetic construct according to claim 1 wherein the circovirus genome comprises more than two DNA components or segments.

3. A genetic construct according to claim 2 wherein the circovirus is subterranean clover stunt virus (SCSV).

4. A genetic construct according to claim 3 wherein the promoter is selected from one of segments 1 to 7 of SCSV as defined by SEQ ID NOs: 1 to 7 or the promoter is capable of hybridizing thereto under low stringency conditions.

5. A genetic construct according to claim 1 or 2 or 3 or 4 further comprising a termination and/or polyadenylation sequence such that said sequence is operably linked to a gene when said gene is operably linked to said promoter.

6. A genetic construct according to claim 5 wherein the termination and/or polyadenylation sequence is of SCSV origin.

7. A genetic construct according to claim 5 wherein the termination and/or polyadenylation sequence is selected from one of segments 1 to 7 of SCSV as defined by SEQ ID NOs: 1 to 7 or the termination and/or polyadenylation sequence is capable of hybridizing thereto under low stringency conditions.

8. A genetic construct according to claim 5 wherein the termination and/or polyadenylation sequence is from the *Flaveria bidentis* MeA gene.

9. A genetic construct according to claim 1 or 2 or 3 or 4 further comprising a heterologous gene operably linked to said promoter.

10. A genetic construct according to claim 5 further comprising a heterologous gene operably linked to said promoter and to said termination and/or polyadenylation sequence.

11. A genetic construct according to claim 6 or 7 or 8 further comprising a heterologous gene operably linked to said promoter and to said termination and/or polyadenylation sequence.

12. A genetic construct according to claim 9 or 10 or 11 wherein the heterologous gene is selected from:

a) a resistance gene against plant viruses, bacteria, fungi, nematode and other pathogens;

b) a plant virus resistance gene including a synthetic gene from and against alfalfa mosaic virus, subterranean clover stunt virus, subterranean clover mottle virus, subterranean clover red leaf virus, potato leafroll virus, tomato spotted wilt virus, bean yellow mosaic virus, white clover mosaic virus, clover yellow vein virus, potato viruses x, y, s, m and a, cucumber mosaic virus, rice ragged stunt virus and barley yellow dwarf viruses;

c) a gene to improve nutritional value of plants such as sunflower high sulphur gene SF8;

d) a bloat resistance gene;

e) an antibody gene;

f) a cereal thionin and ribosome inactivating protein gene;

g) an insect resistance gene including BT toxin gene and proteinase inhibitor gene from *Nicotiana alata*;

h) a selectable marker gene such as those conferring resistance to kanamycin, phosphinothricin, spectinomycin and hygromycin;

i) a reporter gene such as GUS, CAT and pigment genes;

j) a gene encoding a regulatory protein which modulates expression of a gene in plant cells.

13. A genetic construct comprising an SCSV promoter operable in a plant cell, at least one restriction endonucleoase site downstream of said promoter to facilitate insertion of a heterologus gene operably linked to said promoter and optionally a termination and/or polyadenylation sequences located at the 3' end of a heterologous gene when said gene is inserted into said restriction endonuclease site.

14. A genetic construct according to claim 13 wherein the promoter is selected from one of segments 1 to 7 of SCSV as defined by SEQ ID NOs. 1 to 7 or the promoter is capable of hybridizing thereto under low stringency conditions.

15. A genetic construct according to claim 13 or 14 wherein the termination and/or polyadenylation sequence is of SCSV origin.

16. A genetic construct according to claim 15 wherein the termination and/or polyadenylation sequence is selected from one of segments 1 to 7 of SCSV as defined by SEQ ID NOs: 1 to 7 or the termination and/or polyadenylation sequence is capable of hybridising thereto under low stringency conditions.

17. A genetic construct according to claim 13 wherein the termination and/or polyadenylation sequence is from the *Flaveria bidentis* MeA gene.

18. A genetic construct according to claim 13 further comprising a heterologous gene selected from:

a) a resistance gene against plant viruses, bacteria, fungi, nematode and other pathogens;

b) a plant virus resistance gene including synthetic genes from and against alfalfa mosaic virus, subterranean clover stunt virus, subterranean clover mottle virus, subterranean clover red leaf virus, potato leafroll virus, tomato spotted wilt virus, bean yellow mosaic virus, white clover mosaic virus, clover yellow vein virus, potato viruses x, y, s, m and a, cucumber mosaic virus, rice ragged stunt virus and

barley yellow dwarf viruses;

c) a gene to improve nutritional value of plants such as sunflower high sulphur gene SF8;

d) a bloat resistance gene;

e) an antibody gene;

f) a cereal thionin and ribosome inactivating protein gene;

g) an insect resistance gene including BT toxin gene and proteinase inhibitor gene from *Nicotiana alata*;

h) a selectable marker gene such as those conferring resistance to kanamycin, phosphinothricin, spectinomycin and hygromycin;

i) a reporter gene such as GUS, CAT and pigment genes;

j) a gene encoding a regulatory protein which modulates expression of a gene in plant cells.

19. A genetic construct comprising two or more heterologous genes operably linked to the same or different circovirus promoters operable in a plant cell.

20. A genetic construct according to claim 19 wherein the promoter or different promoters are from a circovirus with a genome comprising more than two components or segments.

21. A genetic construct according to claim 20 wherein the promoter or different promoters are from subterranean clover stunt virus (SCSV).

22. A genetic construct according to claim 21 wherein the promoter or different promoters are selected from one of segments 1 to 7 of SCSV as defined by SEQ ID NOs. 1 to 7 or the promoters are capable of hybridizing to at least one of SEQ ID NOs. 1 to 7 under low stringency conditions.

23. A genetic construct according to claim 19 or 20 or 21 or 22 further comprising a termination and/or polyadenylation sequence operably linked to one or more of said heterologous genes.

24. A genetic construct according to claim 23 wherein the termination and/or polyadenylation sequences are the same for each gene.

25. A genetic construct according to claim 23 wherein the termination and/or polyadenylation sequences are different for each gene.

26. A genetic construct according to claim 24 or 25 wherein at least one termination and/or polyadenylation sequence is selected from segments 1 to 7 of SCSV as defined by SEQ ID NOs: 1 to 7 and the termination and/or polyadenylation sequences are capable of hybridizing to at least one of SEQ ID NOs. 1 to 7 under low stringency conditions.

27. A genetic construct according to claim 24 or 25 wherein at least one termination and/or polyadenylation sequence is from the MeA gene of *Flaveria bidentis*.

28. A genetic construct according to claim 19 wherein the heterologous genes are selected from:

a) a resistance gene against plant viruses, bacteria, fungi, nematode and other pathogens;

b) a plant virus resistance gene including a synthetic gene from and against alfalfa mosaic virus, subterranean clover stunt virus, subterranean clover mottle virus, subterranean clover red leaf virus, potato leafroll virus, tomato spotted wilt virus, bean yellow mosaic virus, white clover mosaic virus, clover yellow vein virus, potato viruses x, y, s, m and a, cucumber mosaic virus, rice ragged stunt virus and barley yellow dwarf viruses;

c) a gene to improve nutritional value of plants such as sunflower high sulphur gene SF8;

d) a bloat resistance gene;

e) an antibody gene;

f) a cereal thionin and ribosome inactivating protein gene;

g) an insect resistance gene including BT toxin gene and proteinase inhibitor gene from *Nicotiana alata*;

h) a selectable marker gene such as those conferring resistance to

kanamycin, phosphinothricin, spectinomycin and hygromycin;

i) a reporter gene such as GUS, CAT and pigment genes;

j) a gene encoding a regulatory protein which modulates expression of a gene in plant cells.

29. A method of expressing a foreign gene in a plant cell, said method comprising introducing into said plant cell a genetic construct according to any one of claims 13 to 17 and further comprising said foreign gene operably linked to the promoter on said genetic construct.

30. A method according to claim 29 wherein the foreign gene is selected from:

a) a resistance gene against plant viruses, bacteria, fungi, nematode and other pathogens;

b) a plant virus resistance gene including a synthetic gene from and against alfalfa mosaic virus, subterranean clover stunt virus, subterranean clover mottle virus, subterranean clover red leaf virus, potato leafroll virus, tomato spotted wilt virus, bean yellow mosaic virus, white clover mosaic virus, clover yellow vein virus, potato viruses x, y, s, m and a, cucumber mosaic virus, rice ragged stunt virus and barley yellow dwarf virus;

c) a gene to improve nutritional value of plants such as sunflower high sulphur gene SF8;

d) a bloat resistance gene;

e) an antibody gene;

f) a cereal thionin and ribosome inactivating protein gene;

g) an insect resistance gene including BT toxin gene and proteinase inhibitor gene from *Nicotiana alata*;

h) a selectable marker gene such as those conferring resistance to kanamycin, phosphinothricin, spectinomycin and hygromycin;

i) a reporter gene such as GUS, CAT and pigment genes;

j) a gene encoding a regulatory protein which modulates expression of a gene in plant cells.

31. A method of expressing two or more foreign genes in a plant cell, said method comprising introducing into said plant cell a genetic construct according to any one of claims 19 to 28.

32. A transgenic plant comprising a SCSV promoter and a heterologous gene operably linked thereto and optionally a terminator and/or polyadenylation sequence.

33. A transgenic plant according to claim 32 wherein said heterologous gene is selected from:

a) a resistance gene against plant viruses, bacteria, fungi, nematode and other pathogens;

b) a plant virus resistance gene including a synthetic gene from and against alfalfa mosaic virus, subterranean clover stunt virus, subterranean clover mottle virus, subterranean clover red leaf virus, potato leafroll virus, tomato spotted wilt virus, bean yellow mosaic virus, white clover mosaic virus, clover yellow vein virus, potato viruses x, y, s, m and a, cucumber mosaic virus, rice ragged stunt virus and barley yellow dwarf virus;

c) a gene to improve nutritional value of plants such as sunflower high sulphur gene SF8;

d) a bloat resistance gene;

e) an antibody gene;

f) a cereal thionin and ribosome inactivating protein gene;

g) an insect resistance gene including BT toxin gene and proteinase inhibitor gene from *Nicotiana alata*;

h) a selectable marker gene such as those conferring resistance to kanamycin, phosphinothricin, spectinomycin and hygromycin;

i) a reporter gene such as GUS, CAT and pigment genes.

j) a gene encoding a regulatory protein which modulates expression of a gene in plant cells.

34. A transgenic plant according to claim 32 or 33 wherein the terminator and/or polyadenylation sequence is from SCSV.

35. A transgenic plant according to claim 32 or 33 wherein the terminator and/or polyadenylation sequence is from the *Flaveria bientis* MeA gene.

36. A transgenic plant comprising a genetic construct according to any one of claims 19 to 28.

37. An isolated nucleic acid molecule selected from SEQ ID NO. 1 to SEQ ID NO. 7 or the nucleic acid molecule is capable of hybridizing to at least one of SEQ ID NOs. 1 to 7 under low stringency conditions.

38. A method for enhancing the level of expression of a foreign gene operably linked to an SCSV promoter in a plant cell, said method comprising introducing to said cell a single or two different genetic constructs, comprising a gene to be expressed and a gene whose product is capable of enhancing or transactivating the expression of that gene.

39. A method according to claim 38 wherein the transactivating gene is selected from SEQ ID NO. 1 to SEQ ID NO. 7 or a gene capable of hybridizing thereto under low stringency conditions.

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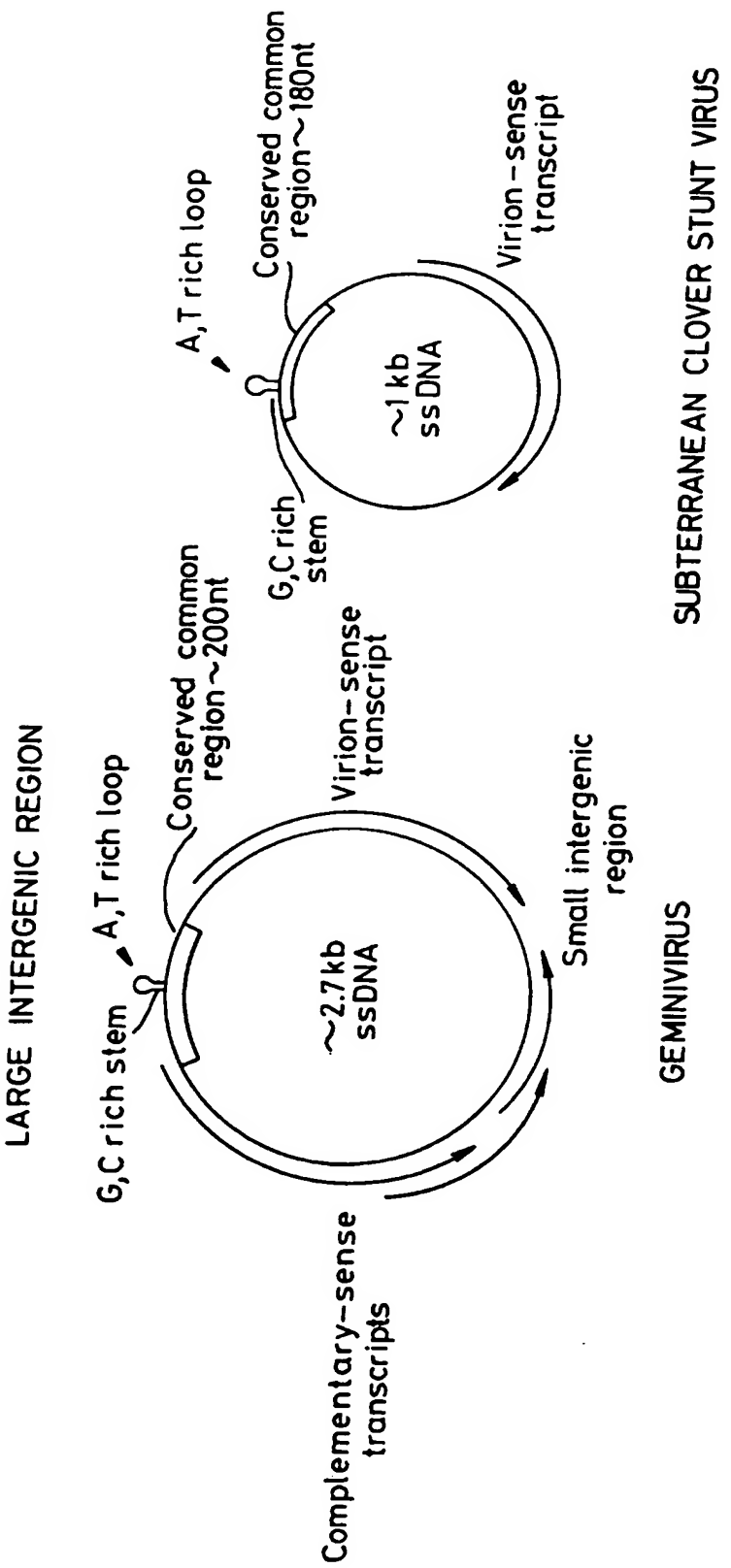
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GEMINIVIRUS
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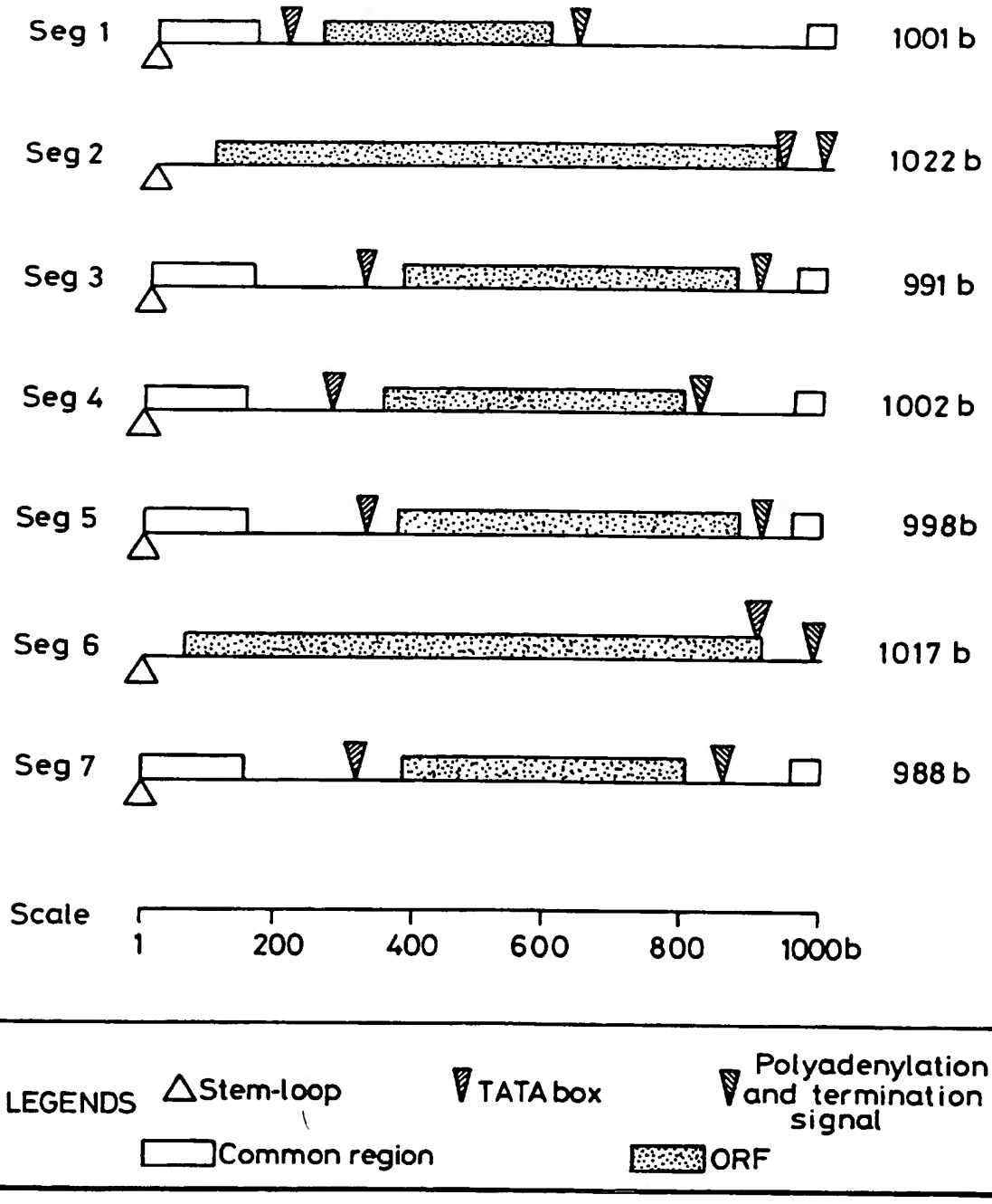
SUBTERRANEAN CLOVER STUNT VIRUS

Fig.1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Stem-loop
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Polyadenylation and termination signal
Common region
ORF

Fig. 2

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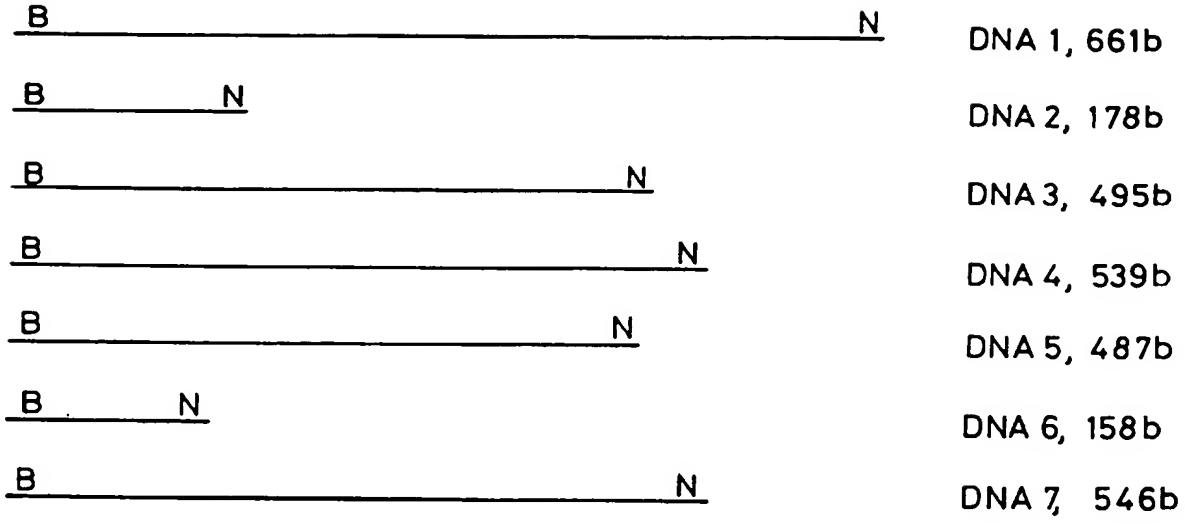
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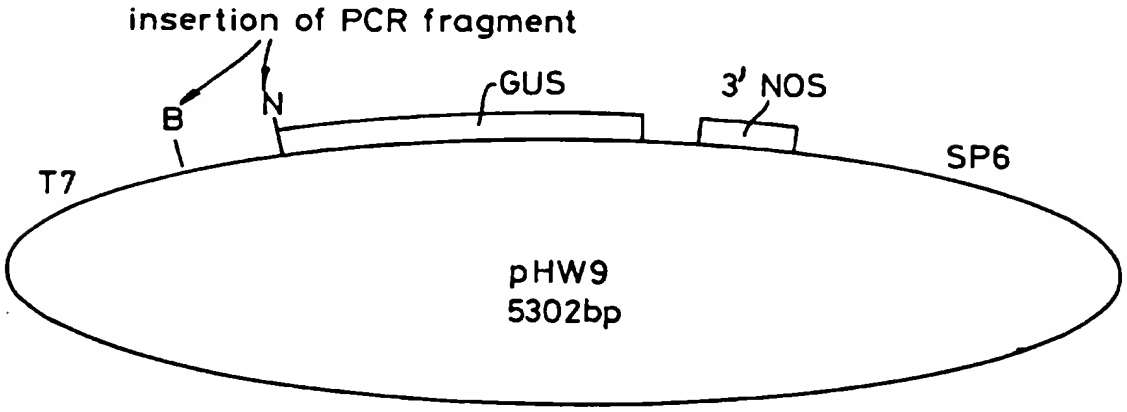
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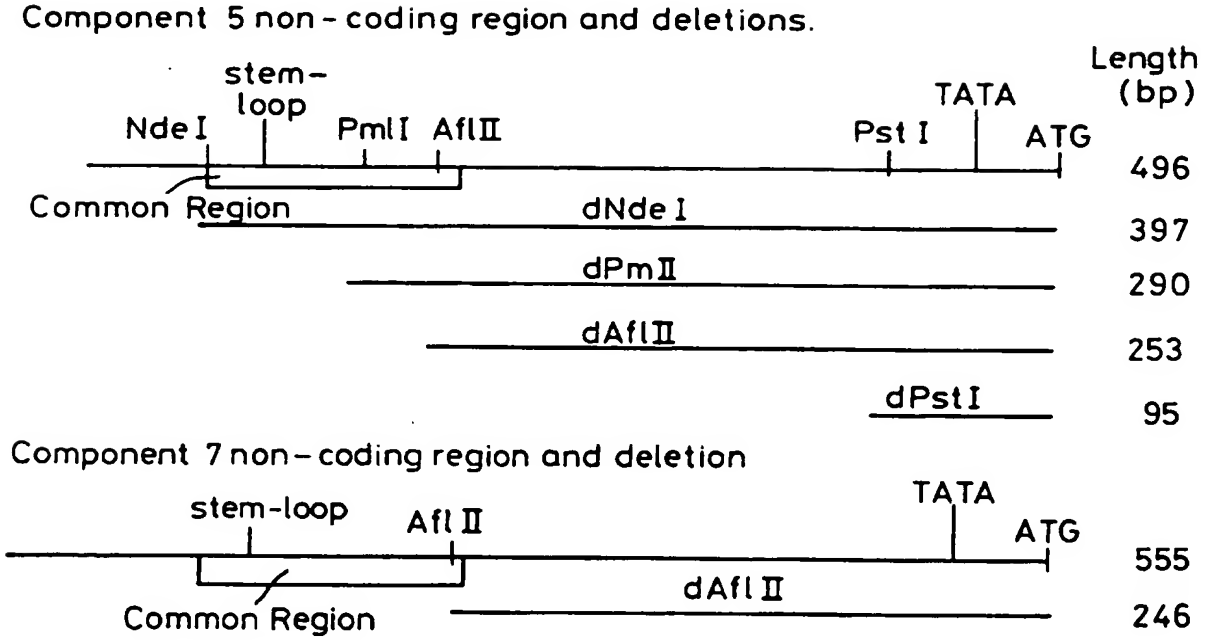
Fig. 3

4/45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dPst I
95
Component 7 non-coding region and deletion
stem-loop
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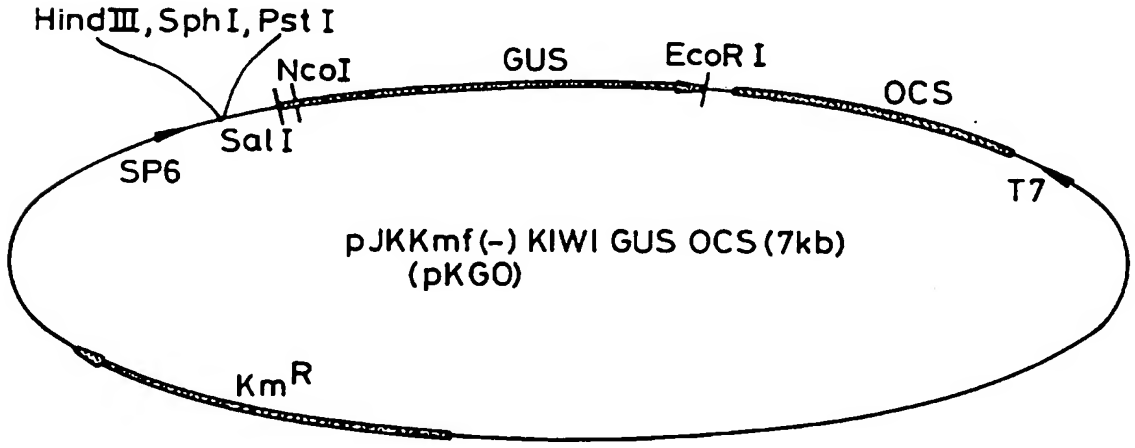
TATA
ATG
555
dAfl II
Common Region
246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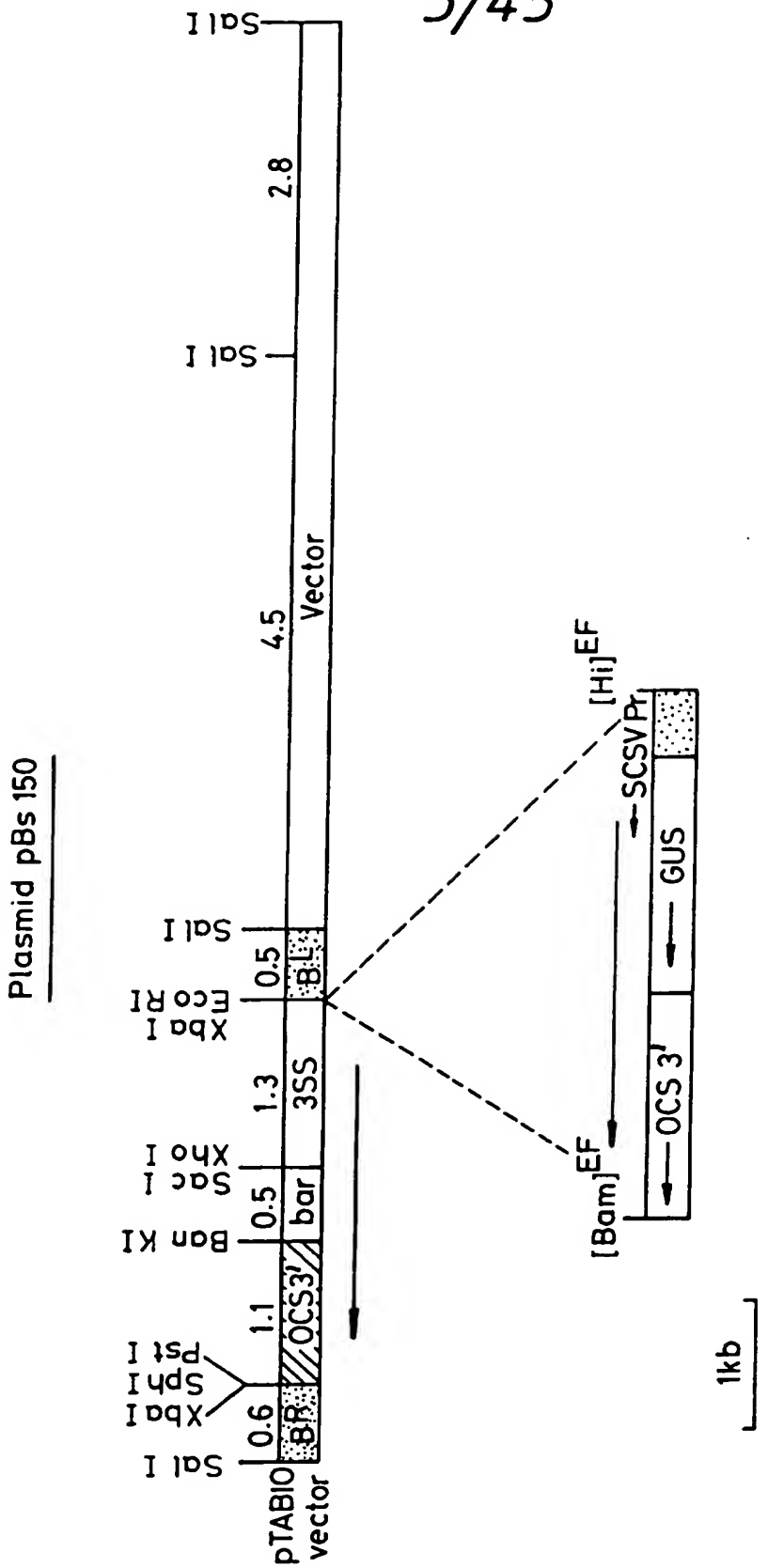
Nco I
GUS
EcoR I
OCS
Sal I
SP6
T7
pJKKmf(-) KIWI GUS OCS (7kb)
(pKGO)
Km R

Fig.4

5/45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2.8
Sal I
4.5
Vector
Sal I
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B
Eco RI
Xba I
1.3
3SS
Xho I
Sac I
0.5
bar
Bgn KI
1.1
OCS3'
Pst I
Sph I
Xba I
0.6
BR
Sal I
pTABIO vector
Plasmid pBs 150
[Hi]EF
SCSVPr
GUS
OCS 3'
[Bam]EF
1kb

Fig. 5

COMPONENT 1

```
1     TAGTATTACC CCCGTGCCGG GATCAGAGAC
61    CCTTGGATTA AATGACACGT GGACGCTCAG
121   GAACGAATCT GACGGAAGAG CGTTCACACT
181   GTCTTGGGTC TATAAATAGA GTGCTTCTGA
241   GATTCTGGTG ATGGTTACAA TACATACTCA
301   GAAGTTTTAT ATAAAATAGG TATTATTATG
361   GTTTTAATTA TATTATGTTG TGCTGTTCCT
421   TTATCTTCGT CTTCTATTAT GAAGAGGAAG
481   GAAGAAACTG GTCCTCATCG TGAAAGAAGA
541   CAGAATAATA ATGATAATGT AAATAGATTT
601   ATGAGAATTA TTATTATTCT GTTCTTCGTC
661   TGGCGTCTGG AGAGAGAAAG GAATAATTGT
721   TGTCTTTACT TCGCCTCGAA GAAAGACACA
781   GAAGCTTCCT CGAAGCAGCG TATAACTTTA
841   ATTGCGGCTG TAAACGTGTC AAGTTGTGAG
901   ATTGTTTTAA TTTAATTCCG CGAAGCGATA
961   GATGACGTCA TATGTCTCCG TGCCTACGTC
```

Fig. 6

COMPONENT 1 (cont.)

ATTTGACCAA TAGTTGACTA GTATAATAGC	60
GATCTGTGAT GCTAGTGAAG CGCTTAAGCT	120
TAGATCTAGT TAGCGTACTT AGTACGCGTT	180
ACAGATTGTT CAGAATTTCA TAGCGAGATG	240
TATGAAGAAG GTGCTGGAGA TGCGAAGAAG	300
TTATGTATTG TAGGGATTGT AGTTTTATGG	360
CGCTATGCTA AATCAACGAT GGACGCTTGG	420
ATGGCTTCAA GGATTACTGG TACTCCGTTT	480
TGGGCTGAAA GAAGAACTGA AGCGACGAAC	540
AGTTGATATG TTGTAATTTT ATATGGATTA	600
TGTGTTTTTT AAGCTTTTTC TGTGTTTTAA	660
AAGGTAGACG ACGATGTAGT GGATTACAGT	720
TTTCAAGTTG TGAGTGTTAT TGCTTTTGAG	780
ATTTGAATTT GGTTTTGGCG CGTTAGTGAA	840
TGGCTGAAAT AAGATAATAG ATATATTATT	900
TGTTAAGTGA TAAATGAAAC GAAGCGTTTT	960
AGCACGGGGC T	1001

Fig. 6 (cont.)

COMPONENT 2

```
1     TAGTATTACC CGACCTTGCC ACACCTCCTT
61    ACTTTCTCTC TCTAAGCTTA TATGGCTAGA
121   GAGATAGAGA GAGAAACATT CCTCTCCCTC
181   GTCGGCGACG AAACTGCAAC TACTGGACAG
241   AACAAAATTC GTCTTGGTGG ATTGAAGAAG
301   GCGAGAGGCA GCGATTCTCA GAATCGCGAT
361   ATTGGGATTC CGGTCATGAA GGGTTCGAAC
421   GATCCCGAAG AAATGCAATT GAAGGATCCA
481   TTGAAAGAGG AATATTGTTC CTGTTATGAT
541   CTTCACGAGG ATTTAATGGC GGAACCAGAT
601   GACGGAGGAG AAGGAAAGAC GAGCTTCGCG
661   ACAGCCGGAG GGAAGACCCA GGACGTATTA
721   ATTGCGTTTG ATGTTCCCAG GTGTTCTTCG
781   TTGAAGAACA GAGTTTTTGC AAGTACAAAA
841   TTAGTTCATT TAATTGTGTT TGCCAACGTG
901   AGACTTGTAA TTATCAATTG TTGAATAAAA
961   GCGAAGCGGT AGCCGGTCAT AACACTGTTG
1021  CT
```

Fig. 6 (cont.)

COMPONENT 2 (cont.)

GGAACACTTT CTCTCTCTAG AAAGTGTGAG	60
AGGTACTGTT TTACATTAAA TTACGCTACT	120
TTCTCTCAAG ACGAATTAAA CTATTTCGTT	180
AAACACCTCC AGGGATTTGT ATCGTTCAAG	240
AAATTTGGTA ATCGAGCTCA CTGGGAAATT	300
TATTGCTGTA AAGAAACCCT AATTTCTGAA	360
AAGCGGAAGA CGATGGAGAT TTATGAAGAG	420
GATACTGCTC TTCGATGTAA GGCGAAGAAA	480
TTTCAGAAAC TCCGTCCATG GCAAATTGAG	540
AAGGAATTAA TCAGGTATGG ATGGTTTTAT	660
TATATGTATG CTCAAGACCC AGAGAGGAAT	720
GAGATGATGA ACTATCAGGC GATGGAGATG	780
TATAGGCCTG TAGATCTTTG TATTAGGAAG	840
GCACCTGACC CCACGCGCAT AAGTGAGGAC	900
GAATATATAT TATTGTTTTA ATTTAATTCC	960
CCCTTGGAAC ACTATATATA GCAAGGTCGG	1020
	1022

Fig. 6(cont.)

COMPONENT 3

```
1     TAGTATTACC CCCGTGCCGG GATCAGAGAC
61    CCCTTGGATT AGATGACACG TGGACGCTCA
121   TGAACGAATC TGACGGAAGA GCGGACATAC
181   TGTATCTCTT TACAGCTATA TTGATGTGAC
241   GTAGGAAATT GCTCGCTAAG TTATTCTTTT
301   ATTAAATGAG TGGCTATAAA TAGATGTTTC
361   TCTTGTGTTA ATGGCGTTAA GGTATTTCTC
421   TATGAACGAG CACTTGAAGG AAATTAAGAA
481   TGCGTGTGCT GTGTTCGAAG GTTTAACAAA
541   ACGCTTCTCT GGGTTTCTGG AAGGTCTGTC
601   GTGTTTAGTT AGATGGAAGA AGAGCGTTGC
661   GATGCATTAC AAGCTTTATG GATTTGCAGA
721   GTTTCCTAAT TACGGTGAAG ACGATGTAGC
781   GCAATTAGAA GTTGTATTTG ATGATTTAGG
841   AGGTTCTATT AAGATAGAAT TATGAGATGT
901   TATTCTTTGA ATTACTCCGC GAAGCGGTGT
961   ATATGTCTCG CCGACAGGCT GGCACGGGGC
```

Fig. 6 (cont.)

COMPONENT 3 (cont.)

ATTTGACCAA TAGTTGACTA TGAATAATAG	60
GGATCTGTGA TGCTAGTGAA GCGCTTAAGC	120
GCACATGGAT TATGGCCCAC ATGTCTAAAG	180
GTAAGATGCT TTACTTCGCC TCGAAGTAAA	240
CTGAAAGAAA TTAATTTAAT TCTAAATTAA	300
GTCTTCGTTG TTTTACAACG AAGCTTAGAA	360
TCATCTTCCT GAAGAACTGA AGGAGAAGAT	420
GAAGGAATTT CTAGAGAATG TAATTAAAGC	480
GAAGGAGTCT GTTGAAGAAG ACGACATACT	540
TGCATATTAT GCAGAGGCGA CGAAGAAGAA	600
AATAAATCTG AAATGGAGAG TTATGGAGGA	660
CATGGAAGAT TTATATTATT CAGAGTTAGG	720
TTATCACGAT GGTGCAATTG TAAATTGTAA	780
TATTGAGTTT ATGTCTATTG TAATTGATAG	840
AATTGTGATT AATGAATAAA GAGTTGTTAT	900
GTTATGTTTT TGTTGGAGAC ATATGACGTC	960
T	991

Fig. 6 (cont.)

COMPONENT 4

```
1     TAGTATTACC CCGTGCCGGG ATCAGAGACA
61    CCTTGGATTA GATGACACGT GGACGCTCAG
121   GAACGAATCT GACGGAAGAG CGGACAAACG
181   AGAAGTGAAT GACAGCTGTC TTTGCTTCAA
241   AAGAATAAGC GTACTCAGTA CGCTTCGTGG
301   GTTGTATCAT CAACGAAGAA GTTAAGCTTT
361   CAGTGCGCTT AAGACATGTA CTCATGTCTG
421   ACAGGATTTC TTCTGTTGTG ATAGTATGCG
481   GTTAGTTAGT TGTTTTGTAA GTTTTACTGG
541   AGGTCAAGTT CAGTTGGGTA TGCAGCAAGA
601   TCCTATTGGG GGTTATTTGT ATCATGATGA
661   CAATCTGGAC ATCGAGTCAG ATTATCTGAA
721   AATTAATATT GTAAATGATA AAGGATTAGA
781   TCATACGATG CGTATTAAGG TGTAATTGTT
841   GTGTTATTTG GTAATTTATG CTTATAAGTA
901   TAATGAGGAT AATAATTGAA TTTGATTAAA
961   TGAGAGTCAC GTGATGTCTC CGCGACAGGC
```

Fig. 6(cont.)

COMPONENT 4 (cont.)

TTTGACTAAA TGTTGACTTG GAATAATAGC	60
GATCTGTGAT GCTAGTGAAG CGCTTAAGCT	120
CACATGGACT ATGGCCCACT GCTTTATTAA	180
GACGAAGTAA AGAATAGTGG AAAACGCGTA	240
CTTTATAAAT AGTGCTTCGT CTTATTCTTC	300
GTTCTGCGTT TTAATGGCGG ACTGGTTTCA	360
TGATTTTTCA GATATTAAGG CGTCTTCACA	420
AGGTAAATTA TCTGAACCTA GGAAGGTGTT	480
TAGTTTTTAT GGAAGTAATA GGAATGTTAG	540
TGATGGCGTT GTTCGTCCAA TAGGATATAT	600
TTATGGATAT TATCAAGGAG AGAAGACGTT	660
GCCTGATGAA GATTTTTGGA AGAGATTTAC	720
TGATAGGTGT GATGTAAAAT GTTATGTAGT	780
ATTATCAATA AAAGAATTTT TATTGTTATT	840
ATTCTATGAT TAATTGTGAA TTAATAAGAC	900
TTAACTCTGC GAAGCTATAT GTCTTTCACG	960
TGGCACGGGG CT	1002

Fig. 6(cont.)

COMPONENT 5

```
1     TAGTATTACC CCGTGCCGGG GTCAGAGACA
61    CCTTGGATTA GATGACACGT GGACGCTCAG
121   GAACGAATCT GACGGAAGAG CGTCATGGTC
181   GTATTGATTT GACTTTACGC GCTTTACTTT
241   TTTGCTCGCG ACGAAGCAAA GTGATTGTAG
301   AACACGGTTT GATTGTGGGT ATAAATATGT
361   ACGAAGATGG TTGCTGTTCG ATGGGGAAGA
421   TCGCGAATTG CTTACAAACC TCCTTCGTCT
481   AATAAGAGAG ATGTTACTGG AGCGGAGGTT
541   ATGAAGAAGG TAATGTTGAT TGCAACATTA
601   CTTATTGTGA AGAGTAATTC GCCTATTGCG
661   TTGATGGTGA AAGAGTCTGT TCAAGATACA
721   TCTTCTGGTA CTGCTGGTAA AGATGTAACT
781   TCAGGTATTA GTCAGACCCA GCATTTGTAT
841   ATCACACTGG AGACGAGAAT GTATATTGAT
901   TTGTTTTTAT TCTTTGAATT ACTCCGCGAA
961   TGACGTCATA TGTCTCCGCG ACAGGCTGGC
```

Fig. 6 (cont.)

COMPONENT 5 (cont.)

TTTGACTAAA TATTGACTTG GAATAATAGC	60
GATCTGTGAT GCTAGTGAAG CGCTTAAGCT	120
CACATGTCTA AAGAATAATG CTTTACAGCT	180
AATTGCTTTA AGTAAAGTAA GATGCTTTAC	240
CTGCAGAAAT TGATGCTTTA ATTACCGGGT	300
TCTGTTCGTT TTCTTCGTTG TCATTTTACA	360
AAGGGTCTGA GGTCTCAAAG GAGAAAATAT	420
AAGGTTGTAA GTCATGTGGA GTCTGTTCTG	480
AAGCCATTCG CTGATGGTTC AAGGTATAGT	540
ACTATGGCTC CTGGAGAATT AGTTAATTAT	600
AATTGGAGTT CGTCTTTCAG TAATCCTTCG	660
GTTACGATTG TTGGAGGAGG AAAGCTTGAG	720
AAGTCTTTTA GGAAGTTTGT TAAGCTGGGT	780
TTAATTATTT ATTCCAGTGA TGCGATGAAG	840
GTATAATTGT GATGATTAAT GAATAAAGAG	900
GCGGTGTGTT ATGTTTTTGT TGGAGACATA	960
ACGGGGCT	998

Fig. 6(cont.)

COMPONENT 6

```
1     CAGTATTACC GCACCTCGCT TACCCTCCTC
61    AAAGCACTAG TTGGGTGTTC ACACTTAACT
121   ATGAAAGCGT TCAGTACGCT TGTTGGCAGC
181   GATTTATACA ATTTAAATCC CGCAACACTA
241   GACTGAATCC TCATCTGGAA ATTGCTAGGG
301   AGGAAGATAG TAGAGTAGCT GGTCCCTGGG
361   ATAAGCGTAA GCTGATGGAG AGATTTGAAG
421   CCTCTCTCTA TAGGCGTTGT CTATCAAGGA
481   AGTGGAATTA TGACTTACGC CCTTGGCAAG
541   CAGATTATAG AACGATAATC TGGGTGTATG
601   TTGCAAGACA TCTGTCATTG AAAGATGGTT
661   ATATGATGCA TCTTGTGACT GCTGAGCCTA
721   TTAGTTCAGA GTATGTGAAT TATGGTGTAA
781   ATACTAAGTA TGAGCCATGT GTAATGCGGG
841   TTGCAAATGT ACTCCCAGAT TTGGGAAAAT
901   GTTGAAAACT CTGCGAAGGC AGAAGTTATA
961   TCGGGTAGTT CGCGAAACAG GGTGAGGGAA
```

Fig. 6(cont.)

COMPONENT 6 (cont.)

GCTTCCCTGG GCCCACTATG CCTACTAGAC 60

TTGAGGGCGA AATTCCTATT TTGCCCTTTA 120

ATGAGAGAGT GGGACACGAT CATTTACAGG 180

CATTGCGTCA GGCTAAGTAT ATTTTTAATG 240

ATGTAGAGAA GGCGCAATTG TACGCGATGA 300

AGTATGGGTT GTTTATTAAG AGAGGATCGC 360

AAGATGGAGA AGAGATGAAA ATTGCTGATC 420

AGATGGCTGA AGAACAACGT TGTTCTTCTG 480

AAGAAGTGAT GCATTTGTTA GAGGAAGAAC 540

GACCTGCTGG TAATGAAGGC AAATCTACAT 600

GGGGTTATCT GCCTGGAGGA AAGACACAAG 660

AGAATAATTG GGTATTTGAC ATACCCAGAG 720

TAGAACAGGT TAAGAATAGG GTAATGGTGA 780

ATGATAATCA TCCTGTTCAT GTAATTGTGT 840

TAAGTGAAGA TAGAATAAAA TTAATTCGTT 900

AAAAAAATGT GTTTTGAGAG AAGTCCCACA 960

GCGAGCAATA TAAGGCGAGG TGCGTAT 1017

Fig. 6 (cont.)

COMPONENT 7

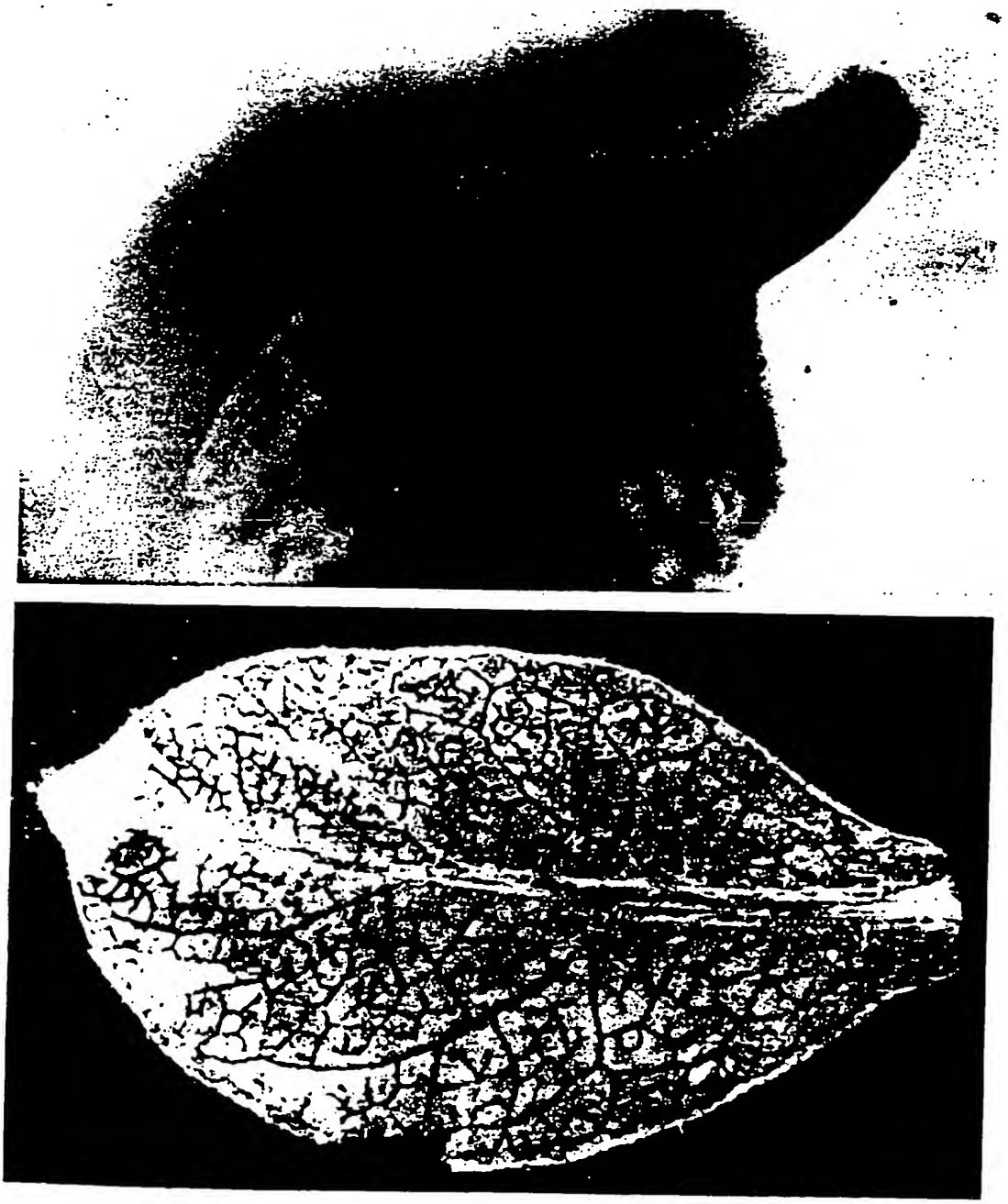
```
1     TAGTATTACC CCGTGCCGGG ATCAGAGACA
61    CCTTGGATTA GATGACACGT GGACGCTCAG
121   GAACGAATCT GACGGAAGAG CGGACATACG
181   GTATCTCTTT ACAGCTATAT TGATGTGACG
241   TAGGAAATTG CTCGCTAAGT TATTCTTTTC
301   TAAATGAGTG GCTATAAATA GTGTCGATGC
361   CTGTTCTGGT TTTAAGCGAT GGTCAGTTTT
421   GATGTTCTTG TAAGCGATAG CAGAAGAAGT
481   GTGATTAACG TGAAGGTACT GAGGTTGATT
541   AAGGTTATGT TTCGTCTGTG TTACAGATAC
601   TGTAAGATGG AGCTATGGAC TTCGTTGAAG
661   TTGCAGAGGA AGCTTAATGG TATATGTGTT
721   GTAAGTAATG TTAAAGAGTT GATTAATAGA
781   AATCCTATAT GTTGTTTGTA TCATATGGAC
841   TATGAGATAA GAGTTGTTAT TAATGCTTAT
901   TTTTATTCGC GAAGCGGTGT GTTATGTTTT
```

Fig. 6(cont.)

COMPONENT 7 (cont.)

TTTGACTAAA TATTGACTTG GAATAATAGC	60
GATCTGTGAT GCTAGTGAAG CGCTTAAGCT	120
CACATGGATT ATGGCCCACA TGTCTAAAGT	180
TAAGATGCTT TACTTCGCTT CGAAGTAAAG	240
TGAAAGAAAT TAATTTAATT CTAATTAAAT	300
TGCCTCACAT CGTATTCTTC TTCGCATCGT	360
AGTTTTCCTG AGATATACGA TGTGAGCGAC	420
GTAGCTGTTG AGGTCGAAGA GAAGGTTCAA	480
GAAGCTGTTG ATGAAGATAG AGTTGGAGTG	540
AGACGAGAAC TGAAGATTAC GTTGTTGGGT	600
TCTTCAGGCA AGTATTCAGT TCAATCTTTG	660
AGTAATTACT GTATAGGTAT TGATATGTTT	720
TGTAAATGGA TTACATCTGT TCAAGGTGTT	780
GAAGAGTAAT TAATAGTAAT TATGATTAAT	840
GAGGAATAAA GAATGATTAA TATTGTTTAA	900
TGTTGGAGAC ATCACGTGAC TCTCACGTGA	960
TGTCTCCGCG ACAGGCTGGC ACGGGGCT	988

Fig. 6 (cont.)



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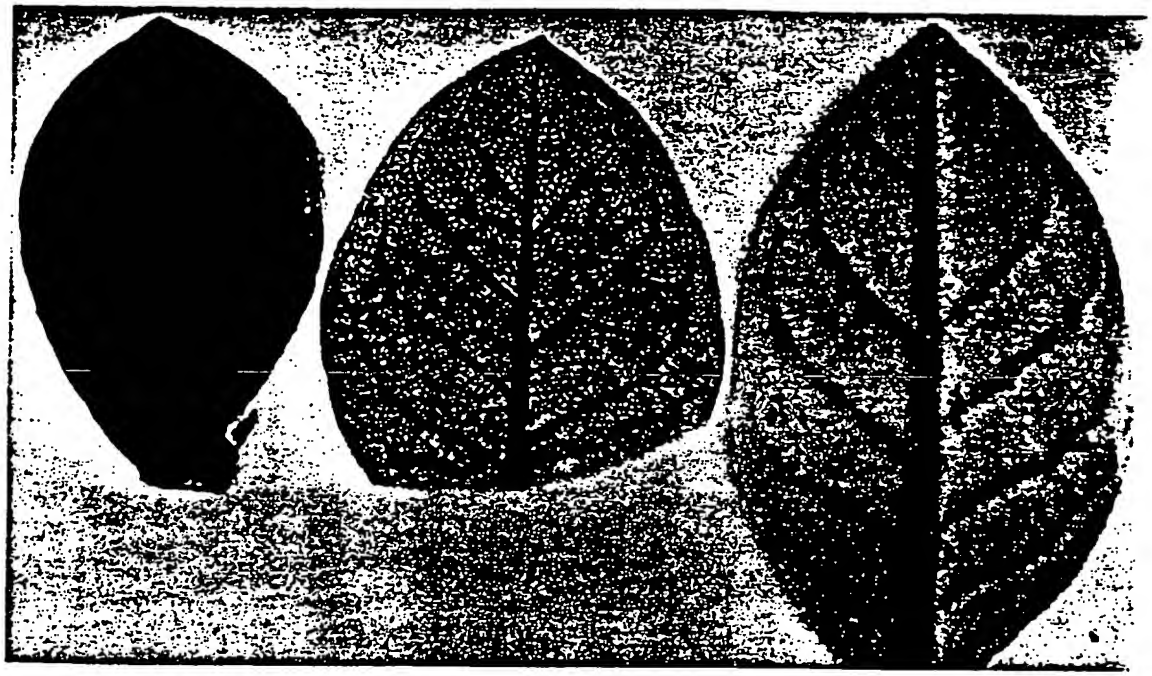

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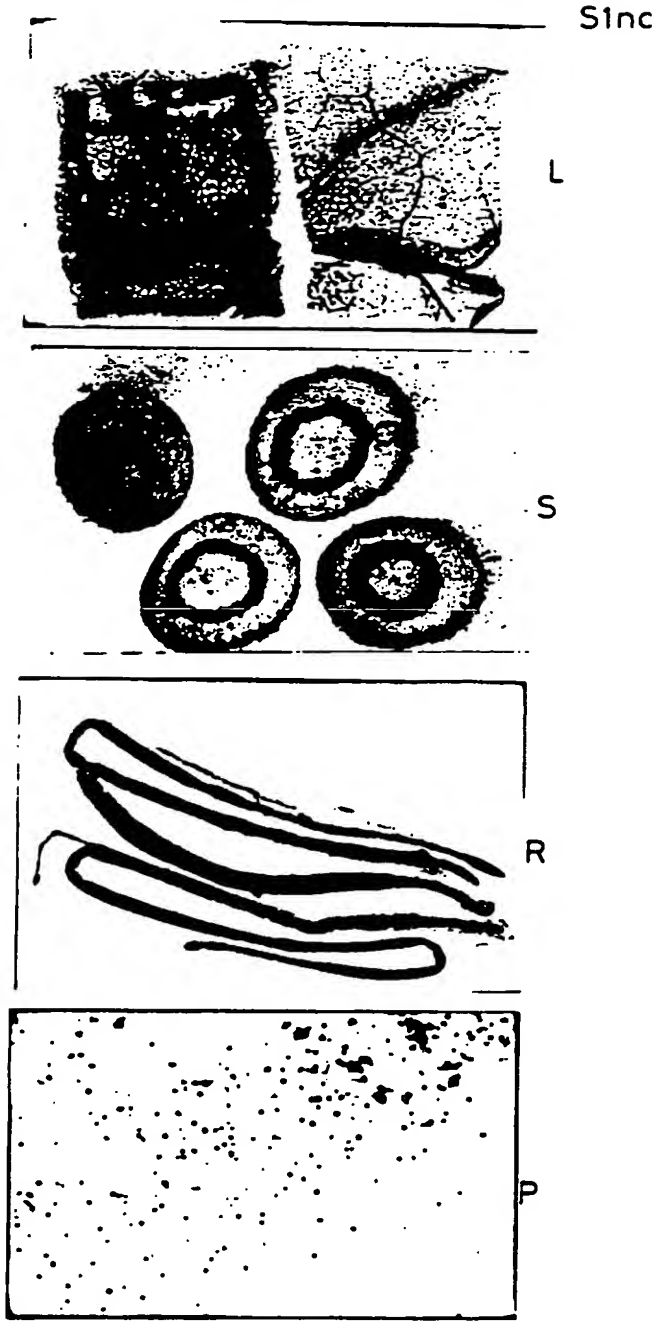


Fig. 8b

23/45

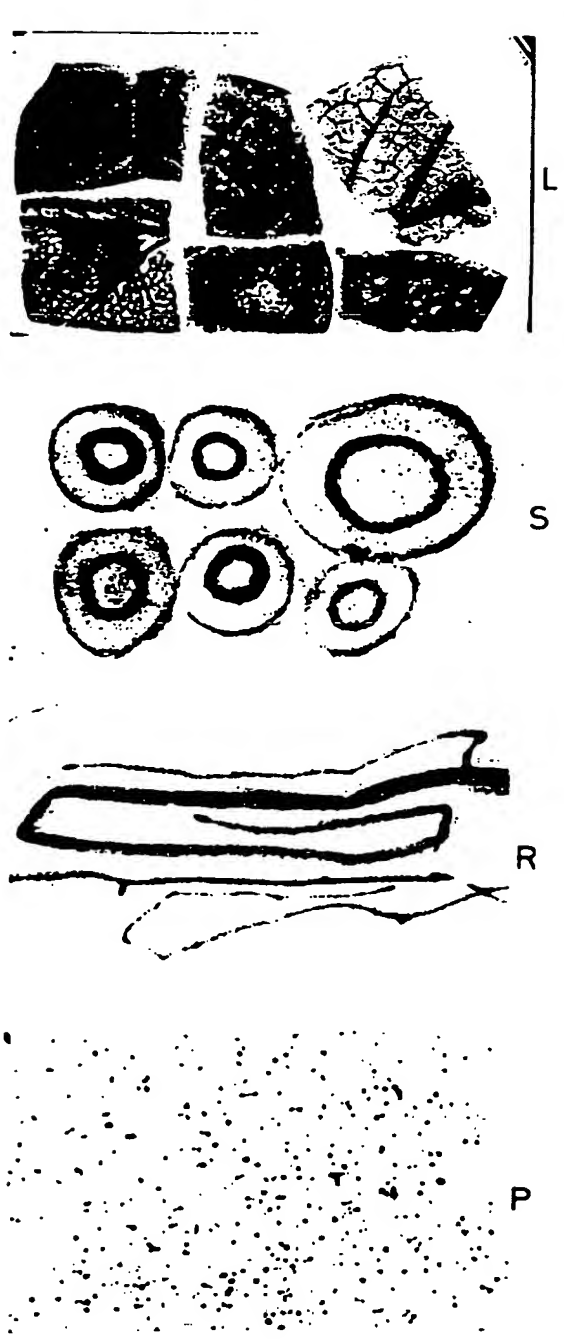


Fig. 8c

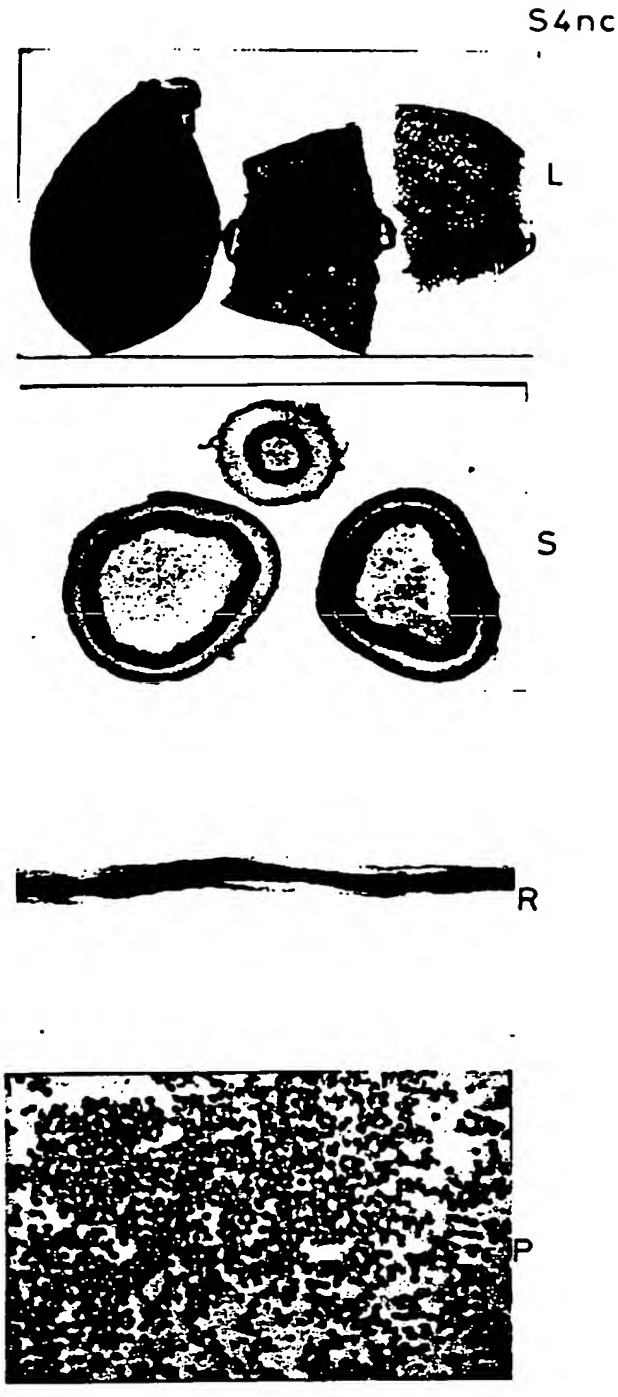


Fig. 8d

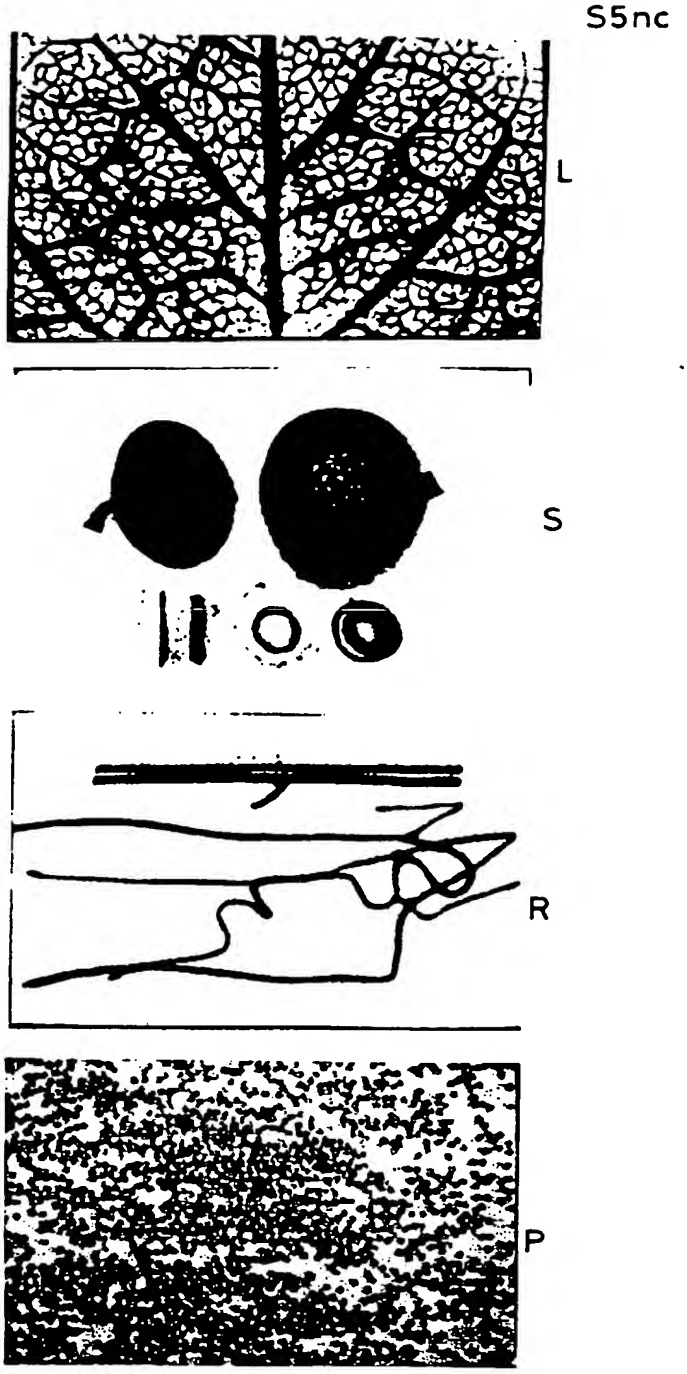


Fig. 8e

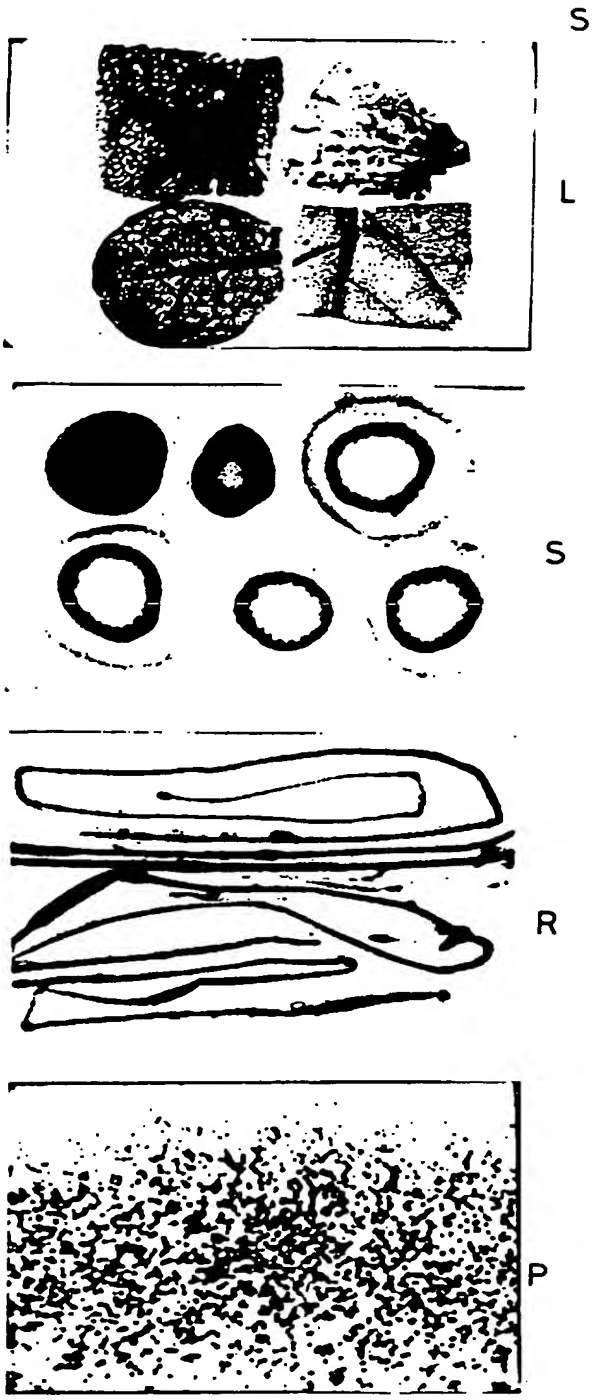


Fig.8f

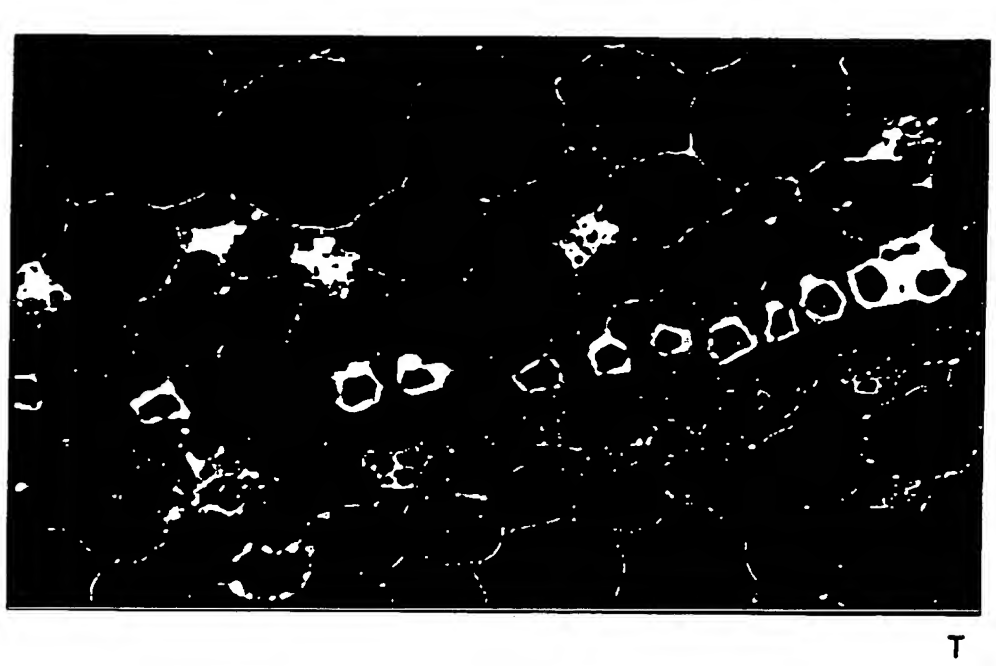


Fig. 9a

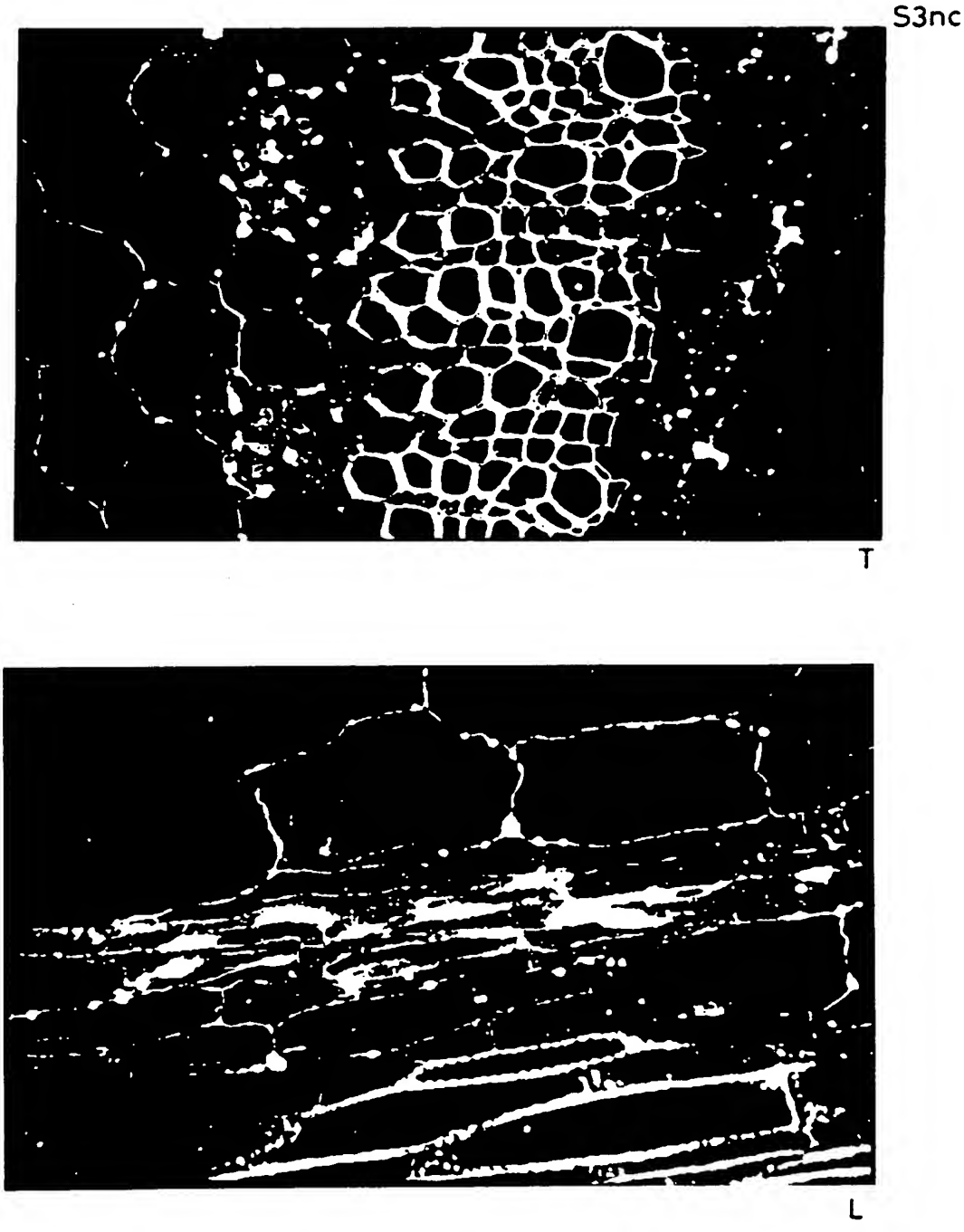
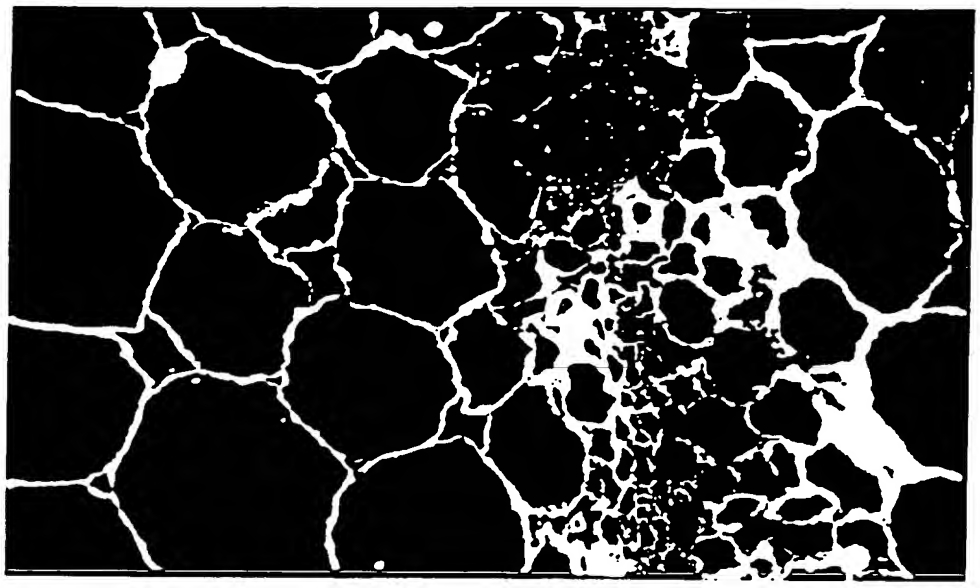


Fig. 9b

S4nc



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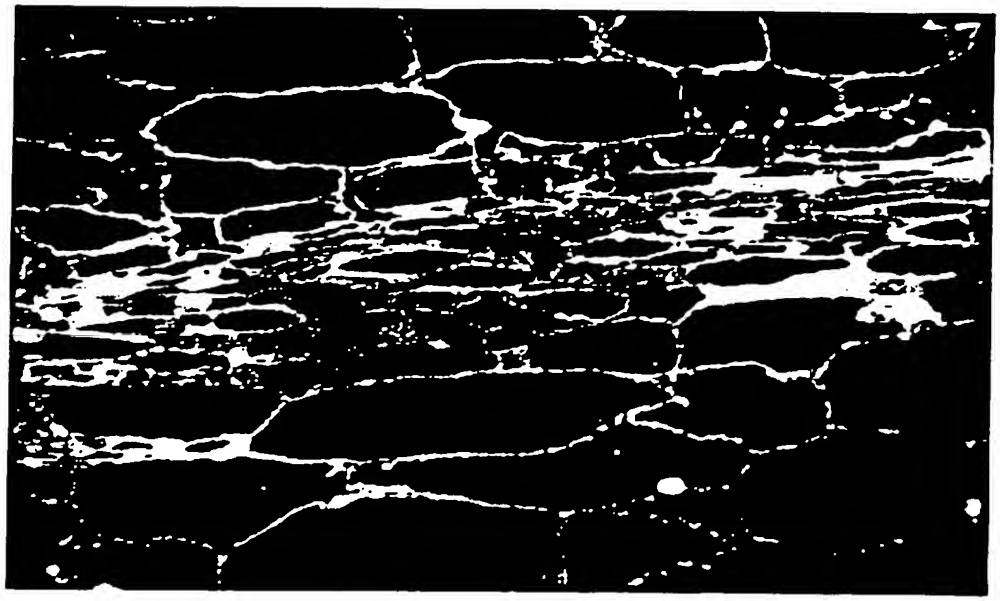
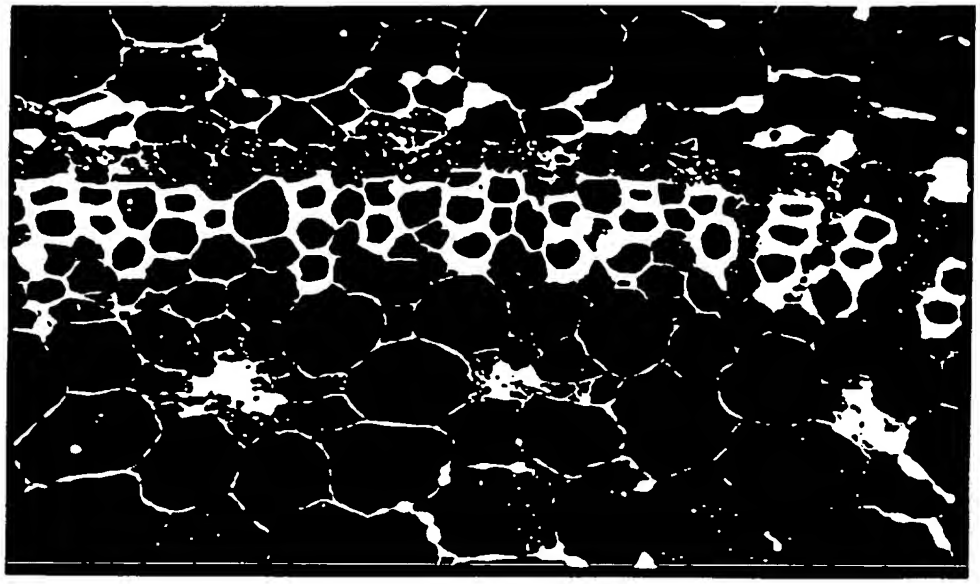


L

Fig. 9c

S5nc



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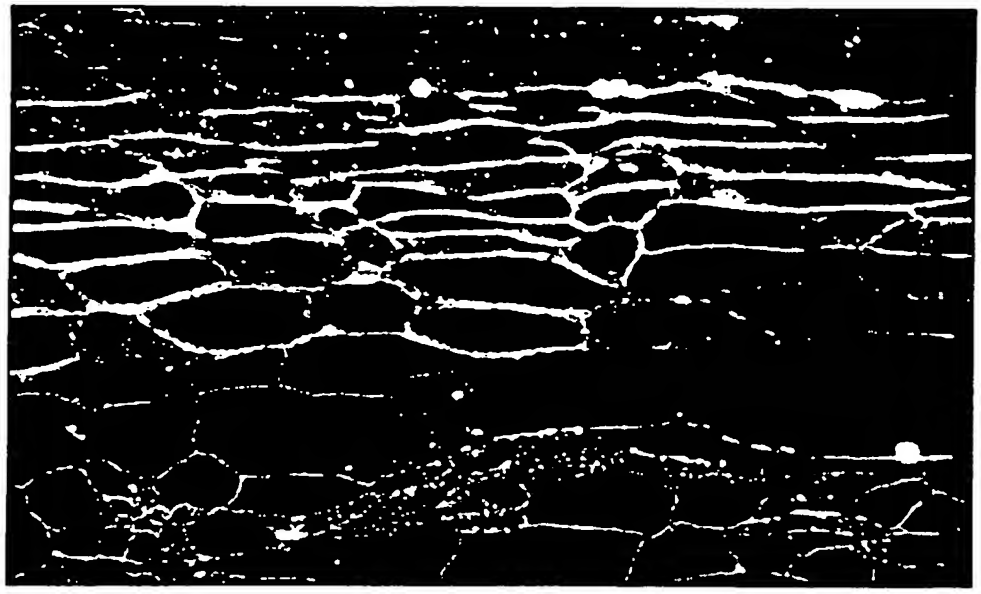

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Fig. 9d

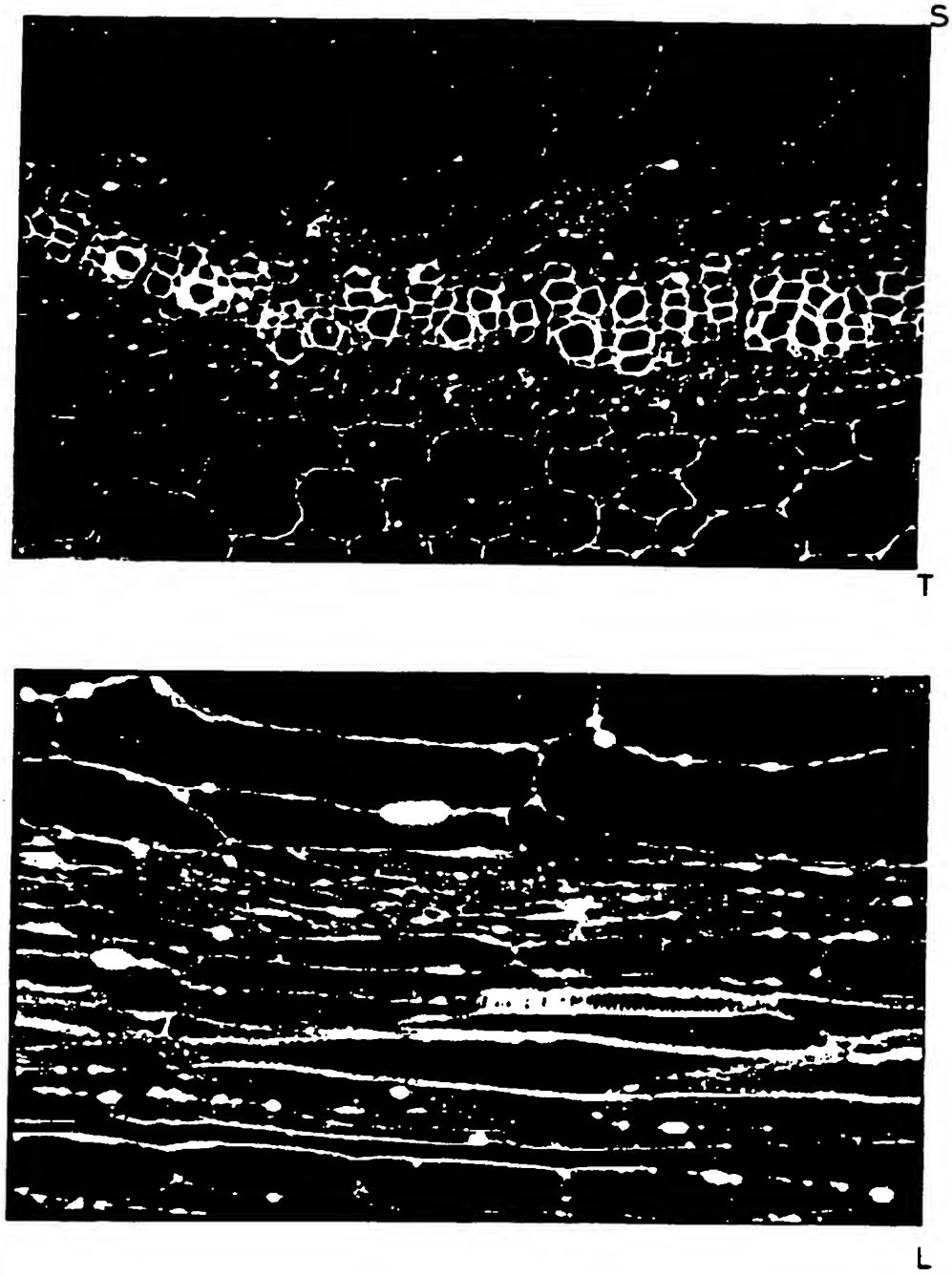
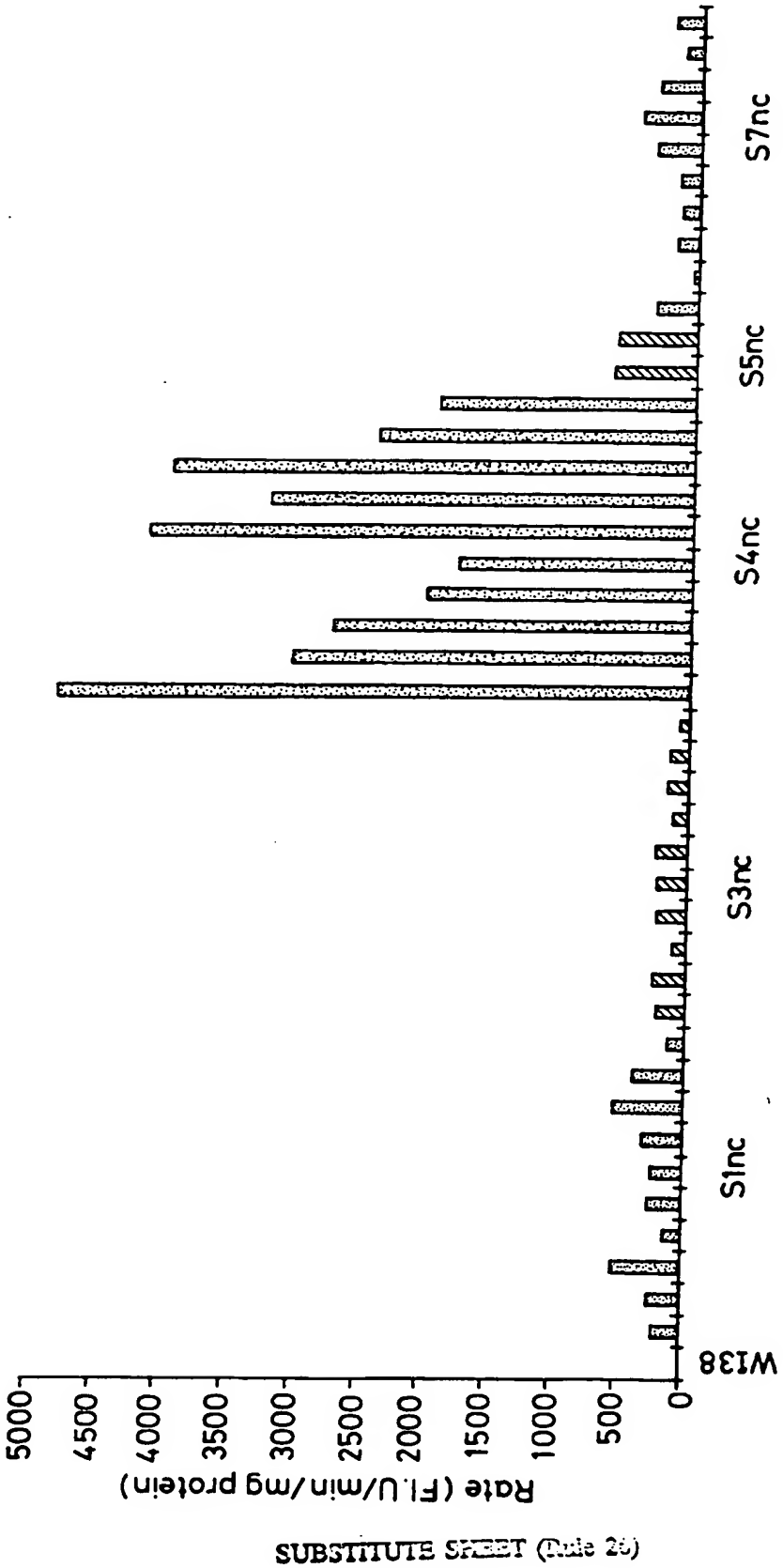


Fig. 9e

32/45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4500
4000
3500
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2500
2000
1500
1000
500
0
W138
S1nc
S3nc
S4nc
S5nc
S7nc

Fig.10

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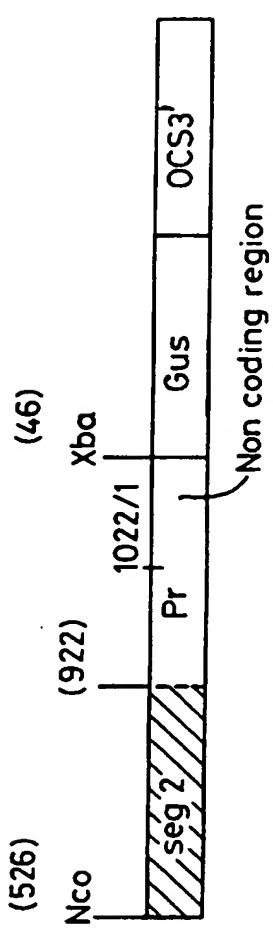


Fig.11

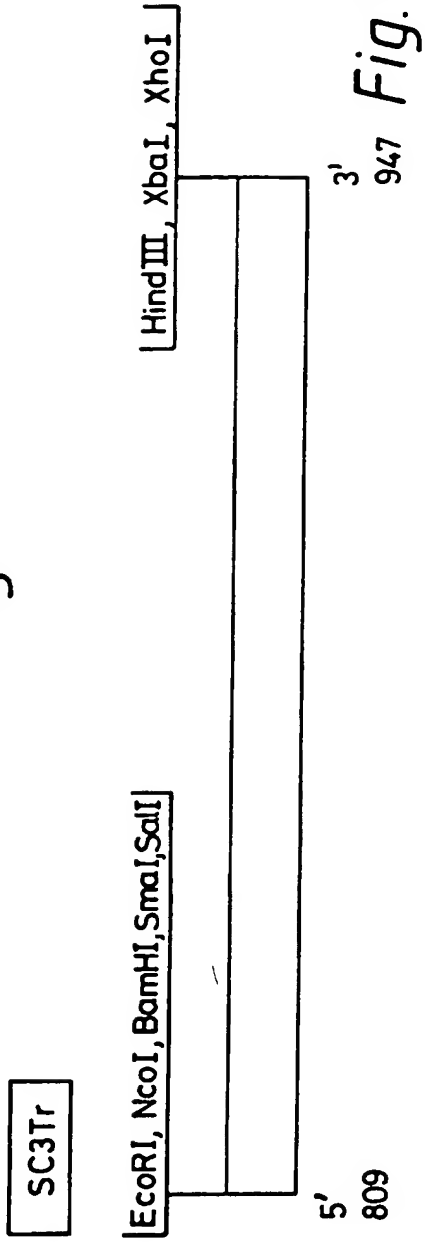


Fig.12a

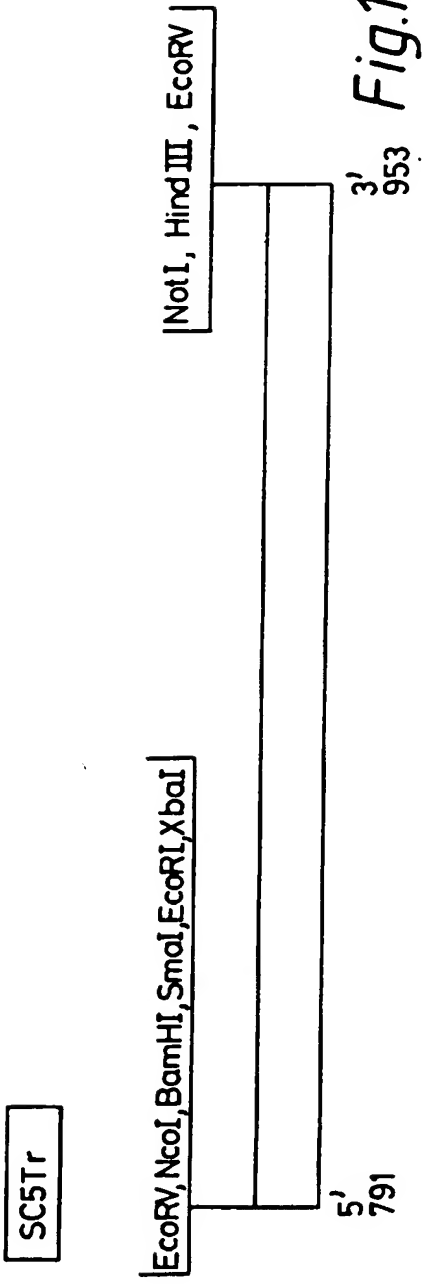


Fig.12b

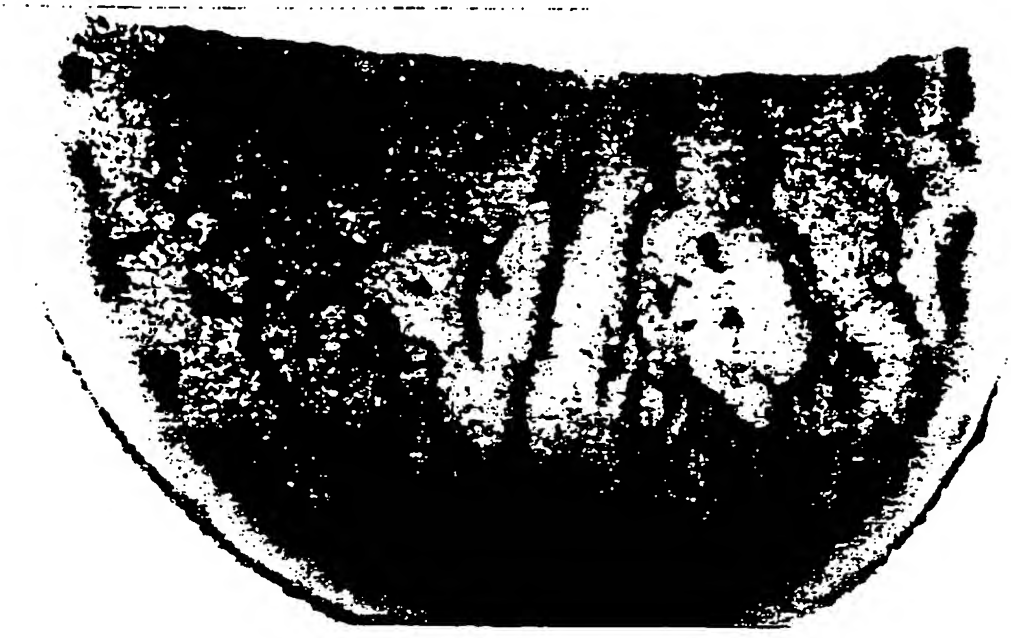
Stem

Fig.13a

Leaf

Fig.13b

Fig.13c Stolon



Tuber

Fig.13d

Fig. 14

```
1     TAAGTTTAGC GGGGGAAAAA GGACAGTTGA
61    ATGTTGTCAG ATGCATGTTG TAATGCTTGT
121   TGATGATGGA AACTTAAAGC TTAATACTAC
181   GGTTCTTTGT TTATCAGGAA TGCTCATTGT
241   TATTGCCCTA AATCTGGTAC TTTATCCAAA
301   TCAAGAATGA TAAACTCGTA CACTCTCTAG
361   AACCGCTTAG GAAGGAACAT ATGTGATAAG
421   AGTCGGCCCA ATTCGAGAGG ACTAGTCTCC
481   TAAACAAAAC ACATATAAAA AACCTAAAAA
541   GTGGGAACAG TTACAAATCT GCAGTCTCAC
601   CAAGTTTTCG AATGTTCTCC CACCATTCAC
661   TAGCTGACAC AACCCGTTTT GACCCAACAT
721   AATCGACTTG TCCTCAAGTC GAAAGGAGGG
781   ATTGGAGGTT GATGGATGAT TTCCTTGTGT
841   GCTTTTAATG CCTCTTGAAC TGTAGCCACA
901   AATTC
```

Fig. 15

```
TCTGTTGCTG TTTGCAATTT TTTAAAGGGT     60
TCATCAACAC ATTATATGAC TTGCAGTTGC    120
TTTTGTTTAT TCACTTACAA ATACCGGTTG    180
ATGTAGCTAA AAGCTGGCCG TTTATAGTTT    240
AACTAAATTT GGAAACATCA AATACTTTTT    300
GGTACTCCTG AAATTTAAAT CAAAATCCAA    360
AACTGAAATT TCGATTAACT ATTACAAGAT    420
GATTACAAGG AGTAAATATC TTAATCTTGA    480
TATAGGAACA TAATACATAA ACTAAAAGTT    540
TCCCTAAATT TGTGAGTCAC CTTTCACCTA    600
TTTCCCTCCA CCCGGATTCC CTCCAATTAA    660
TGGGTTCGTA TCAATACATC CGGCCCGGAA    720
GAATTATTGT GCCAAGCAAA AAGCCATTCG    780
TTGAAAGCTT CAAAAGATCC GGCCAAATCA    840
ACACCACTTT GAAACCTCAA ATCTGTTTTG    900
                                    905
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Fig.15 (cont.)

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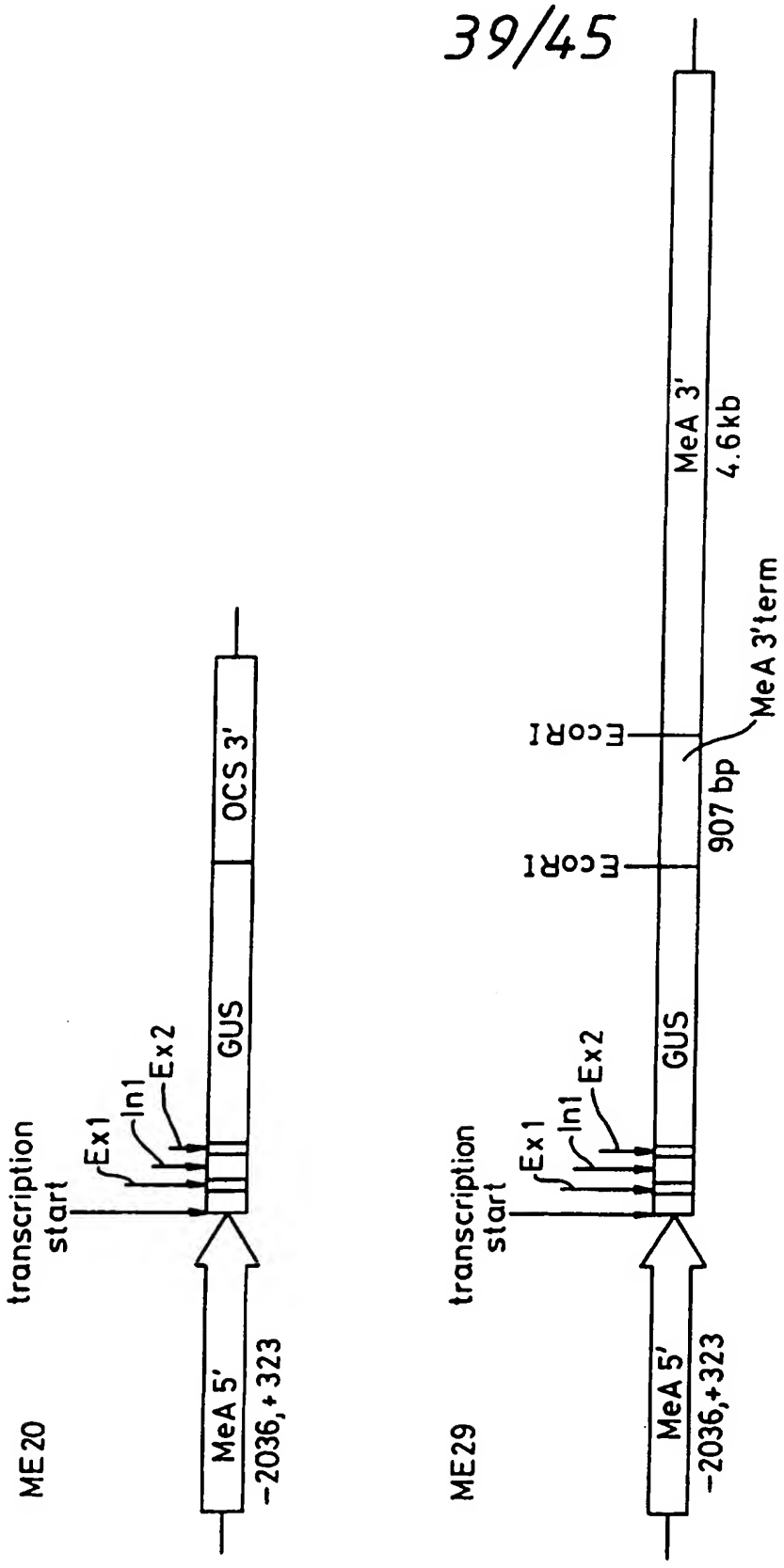
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transcription start
Ex1
In1
Ex2
GUS
OCS 3'
MeA 5'
-2036,+323
ME29
transcription start
Ex1
In1
Ex2
GUS
EcoRI
907 bp
EcoRI
MeA 3'term
MeA 3'
4.6kb
MeA 5'
-2036,+323

Fig. 16

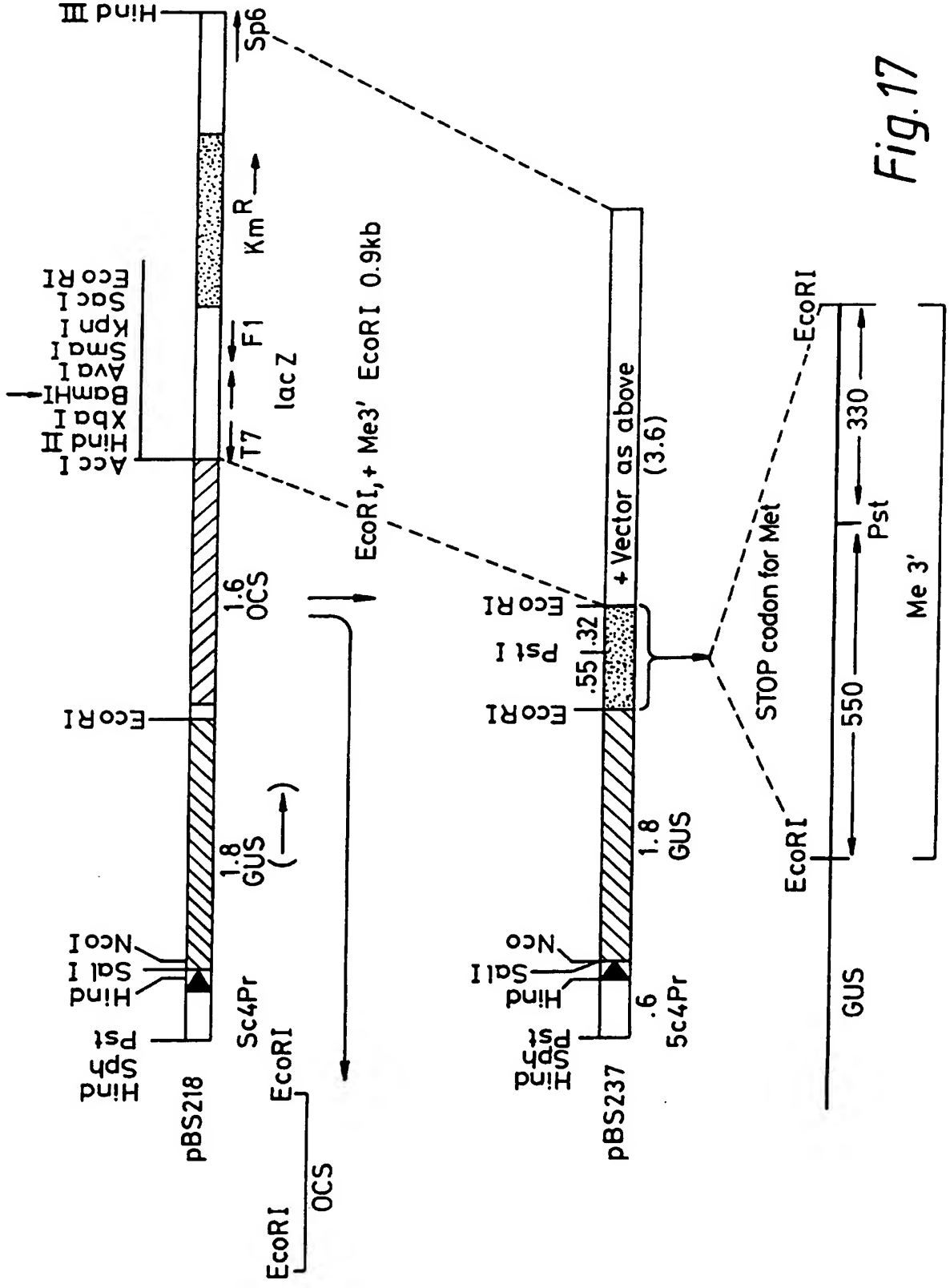
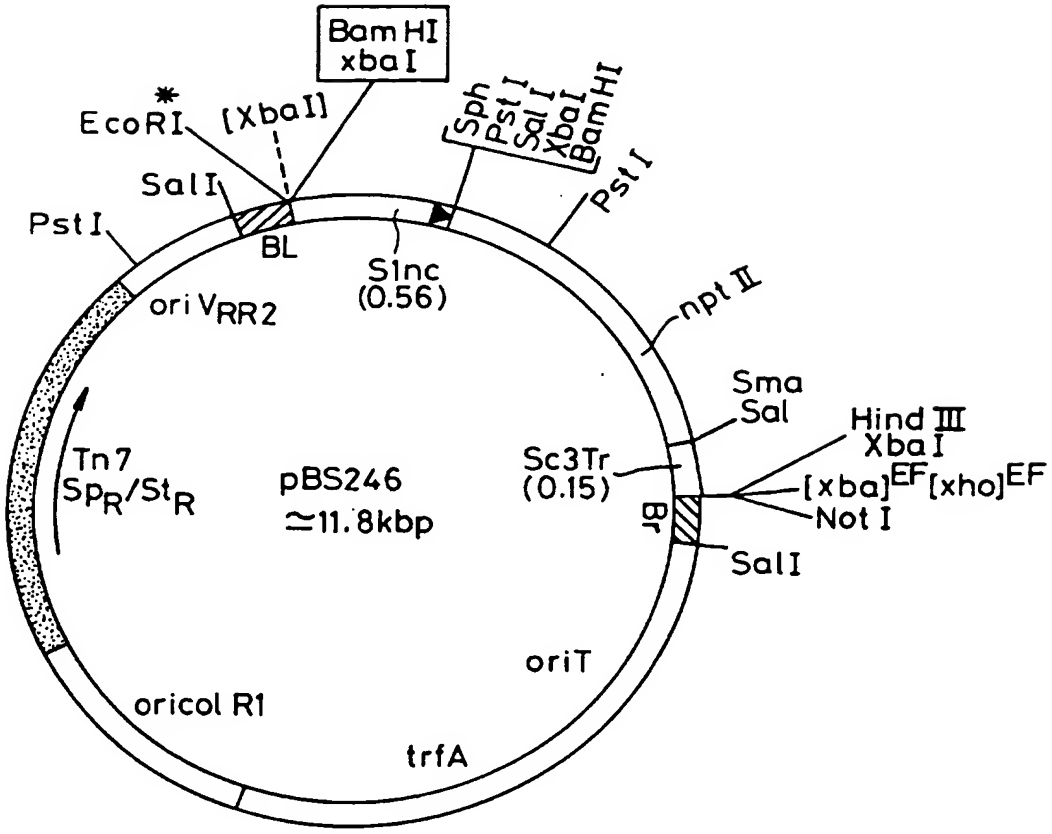

Hind III
Sp6
Km R
F1
lac Z
T7
Acc I
Hind III
Xba I
BamHI
Ava I
Sma I
Kpn I
Sac I
Eco RI
EcoRI, + Me3' EcoRI 0.9kb
1.6
OCS
Eco RI
1.8
GUS
Nco I
Sal I
Hind
Pst
Hind
Sph
Sc4Pr
pBS218
EcoRI
OCS
EcoRI
+ Vector as above
(3.6)
Eco RI
Pst I
.55
.32
Eco RI
1.8
GUS
Nco
Sal I
Hind
Pst
Hind
Sph
.6
5c4Pr
pBS237
STOP codon for Met
EcoRI
330
Pst
550
EcoRI
GUS
Me 3'

Fig.17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xbaI
EcoRI
[XbaI]
Sph
PstI
SalI
XbaI
BamHI
PstI
SalI
PstI
BL
Sinc
(0.56)
oriV RR2
nptII
Sma
Sal
Hind III
XbaI
[xba]EF [xho]EF
Not I
Tn7
SpR/StR
pBS246
≃11.8kbp
Sc3Tr
(0.15)
Bt
SalI
oriT
oricol R1
trfA

Fig. 18

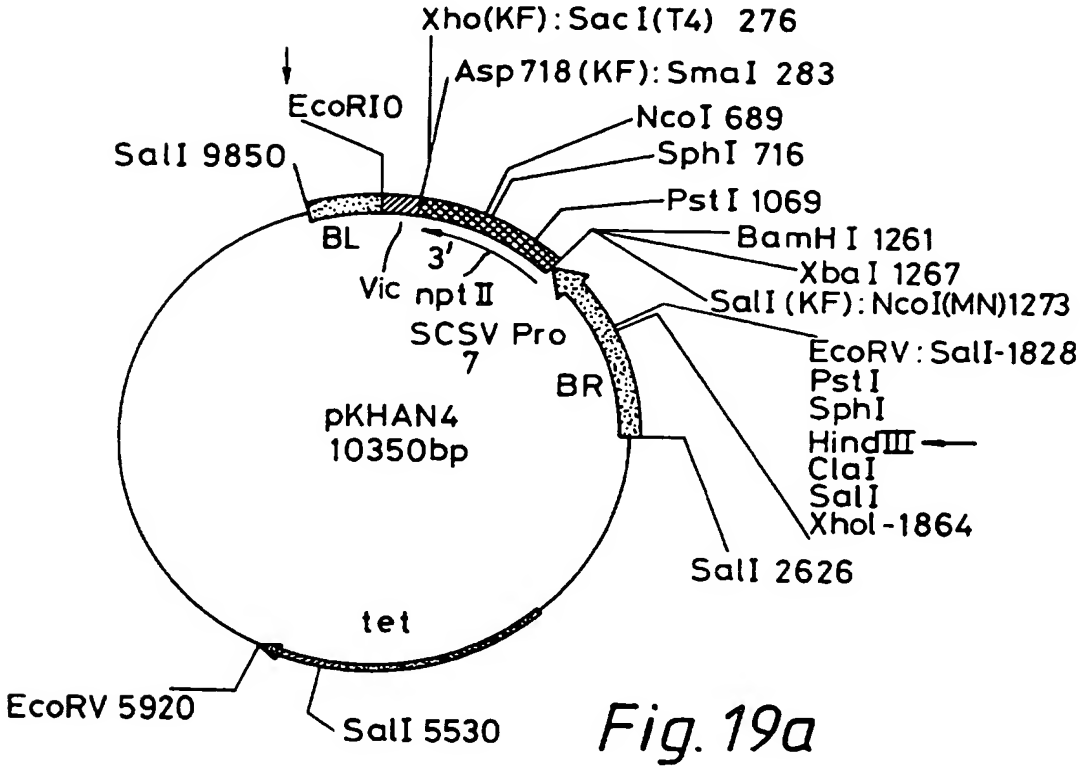

Fig. 19a

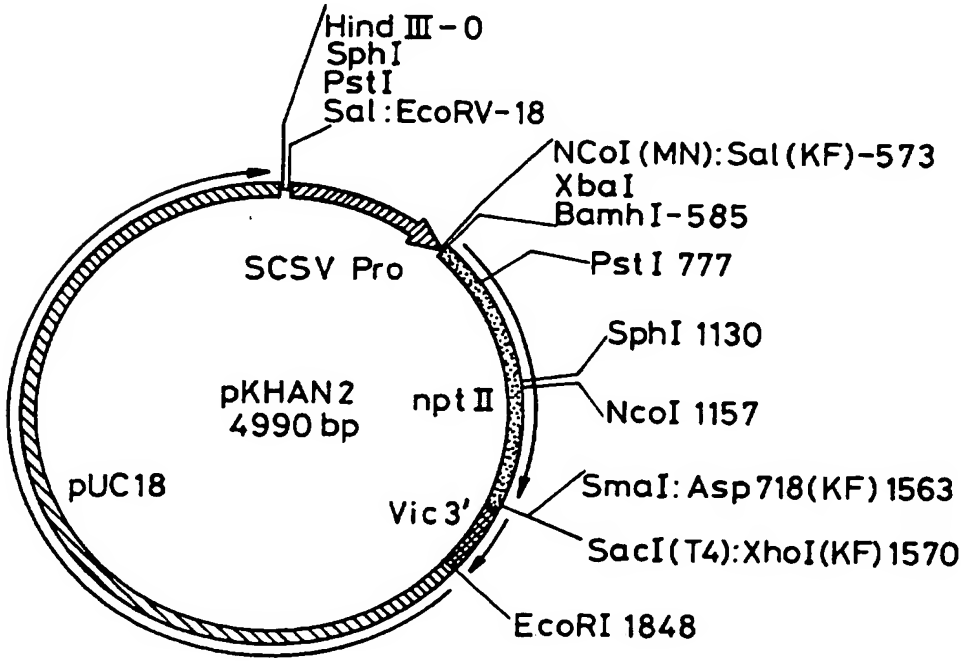

Fig. 19b

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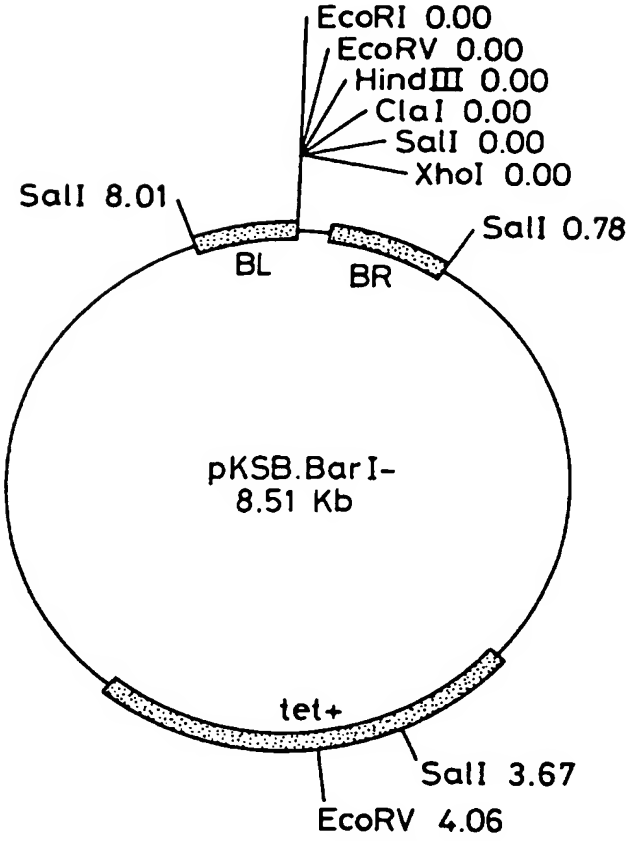

Fig. 19c

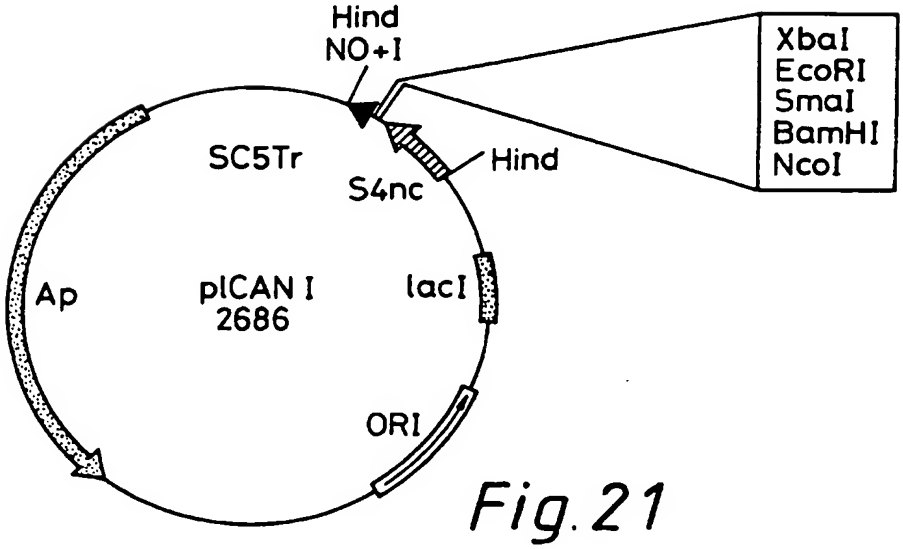

Fig. 21

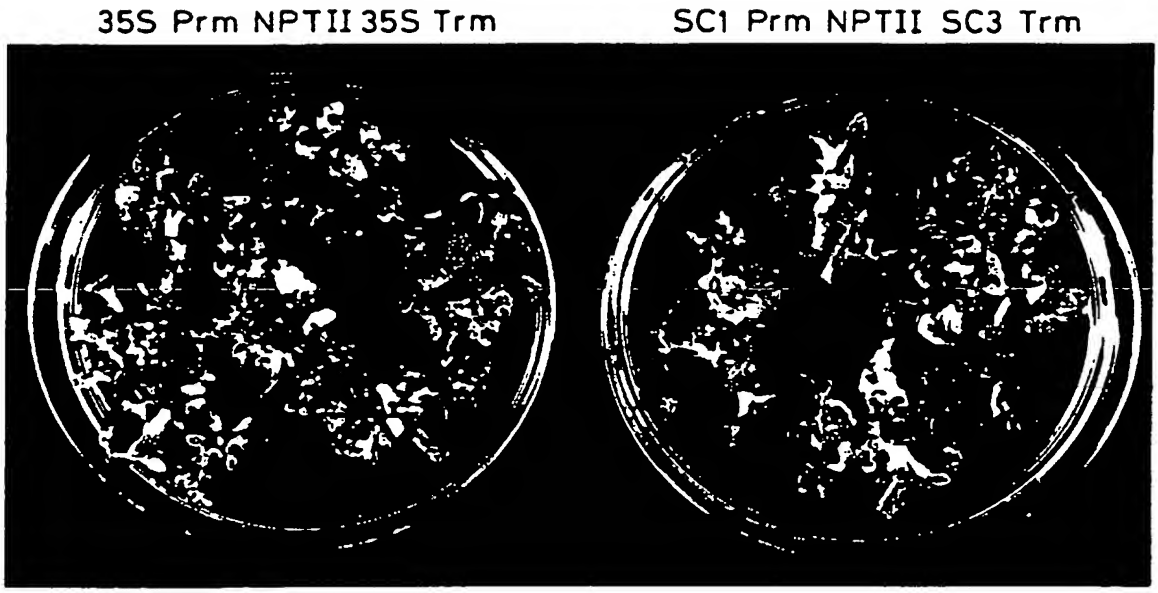

Fig. 20

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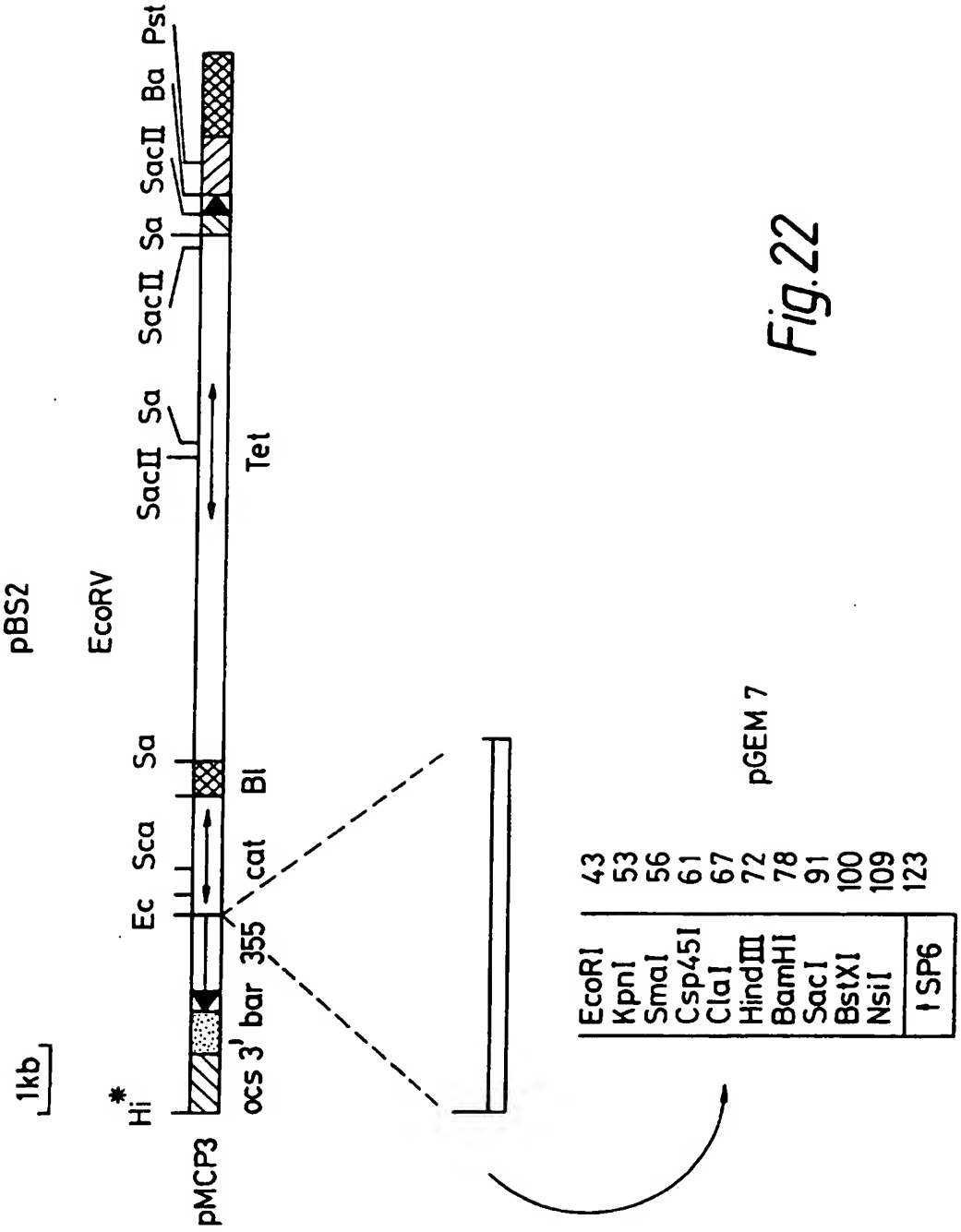
pMCP3
Hi
*
1kb
ocs 3'
bar
355
cat
Ec
Sca
Sa
Bl
pBS2
EcoRV
SacII
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Sa
SacII
Ba
Pst
Tet
pGEM 7
EcoRI 43
KpnI 53
SmaI 56
Csp45I 61
ClaI 67
HindIII 72
BamHI 78
SacI 91
BstXI 100
NsiI 109
SP6 123

Fig.22

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 95/00552

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C12N 15/11 15/83, A01H 5/00 5/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See electronic data base box below

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
BIOTECHNOLOGY ABSTRACT (BIOT) see electronic data base box below

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Derwent databases - WPAT, JAPIO, USPM and BIOT - the following key words were used in the online search: CIRCOVIRUS## or CIRCO () VIRUS## or SUBTERRAN: () CLOVER () STUNT VIRUS## or SCSV or COCONUT (5W) FOLIAR () DECAY () VIRUS## or CFDV or BANANA () BUNCHY () TOP () VIRUS## or BBTV or (MILK () VETCH or MILKVETCH) () DWARF () VIRUS## or MDV or FABA () BEAN () NECTROTIC () YELLOW () VIRUS## or FBNYV

continued

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE, 4306832 (Max-Planck Gesellschaft zur Förderung der Wissenschaften) 24 February 1994: whole document especially page 2 lines 55 - page 3 line 18 examples I.2 - examples II.3 and figures 1-3	1-2, 5, 9-12, 19, 28-31, 36
P,X	Virology volume 207 pages 354-361 (1995) P. Boevink et al "Sequence of subterranean clover stunt virus DNA: Affinities with geminivirus". See pages 356-357 and figures 1 and 2	1-7, 19-22, 37

[X] Further documents are listed in the continuation of Box C

[ ] See patent family annex

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 December 1995	7 DECEMBER 1995
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (06) 285 3929	Authorized officer **J.H. CHAN** Telephone No.: (06) 283 2340

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 95/00552

C (Continuation) **DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Journal of General Virology (1995) volume 76, pages 574-479, Katul Lina et al "Sequence analysis of a Faba bean necrotic yellow virus DNA component containing a putative replicase gene". See especially pages 476-477 figures 1 and 3	1
P,X	Journal of General Virology (1994) volume 75, pages 3541-3546 Karan Mirko et al "Evidence for two groups of banana bunchy top virus isolates". See especially figure 1, page 3544, lines 14-23	1
X	Phytopathology volume 84, no. 9 issued 1994, pages 952-958 WU R.Y et al "Nucleotide sequences of two circular single-stranded DNAs associated with banana bunchy top virus. See especially page 956	1
P,X	Phytopathology volume 85, no. 3 (1995), Xie W.S. and Hu J.S. "Molecular cloning sequence analysis and detection of banana bunchy top virus in Hawii". See page 344	1
A	Virus Research Volume 27 (1993) pages 161-171 Chu Paul W.G. et al "Putative full-length clones of the genomic DNA segments of subterranean clover stunt virus and identification of the segment coding for the viral coat protein".	
A	Virus Research volume 27 (1993) pages 173-183 Chu Paul W.G. et al "Replication of subterranean clover stunt virus in pear and subterranean clover protoplasts"	

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 95/00552

Box B FIELDS SEARCHED - Electronic data base (continued)

STN database - Chemical abstracts (CAS ONLINE):

(1) The following key words were used in the online search:

(CIRCOVIRUS? or CIRCO()VIRUS? or CLOVER()STUNT()VIRUS? or FOLIAR()DECAY()VIRUS? or ?VETCH()DWARF()VIRUS? or BANANA()BUNCHY()TOP()VIRUS? or BEAN()NECROTIC()YELLOW()VIRUS?) AND 3/CC

(2) The following nucleic acid sequences have been searched as fragments together with the specified key words:

(TACATACTCA or TACTGGACAG or TACAGCTATA or TACTCAGTAC or GATTGTGGGT or TAGAGTAGCT or GCTATAAATA) and PROMOTER#/IT and 3/CC and not P/DT.

(3) The following nucleic acid references have been searched as fragments:

GTTGTCTTGGGTCTATAAA; TATCAATTGTTGAATAAA; TTAAATGAGTGGCTATAAA; CTTCGTGGCTTTATAAA; TTGATTGTGGGTATAAA; TGAAGATAGAATAAA; TAATGAGTGGCTATAAA.

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